Interaction of enterohaemorrhagic *Escherichia coli* (EHEC) with bovine intestinal epithelium

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Declaration

I declare that this thesis and the experiments described therein are my own work, except where otherwise indicated. No part of this thesis has been submitted for a degree at this or any other university.

Arvind Mahajan
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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC), particularly of serotype O157:H7, are the most common cause of haemorrhagic colitis (HC) which can lead to life-threatening haemolytic uraemic syndrome (HUS) in humans. Verotoxins (VT; or shiga-like toxins – Stx) are major virulence factors and are key determinants in the patho-physiology of EHEC infections in humans. Cattle are the most important asymptomatic reservoir host for EHEC and recently terminal rectum was identified as the principal site of *E. coli* O157:H7 colonization in cattle. First objective of this research project was to immuno-phenotypically characterise terminal rectal tissue of cattle. Secondly, to develop and characterise primary rectal epithelial cell culture as an *in vitro* model to study interaction with EHEC strains. The primary epithelial culture with mixed population of cells including proliferating crypt-like, differentiated epithelial and goblet cells, closely resembled a native intestinal epithelium. A subset of cells in the culture expressed vimentin and trancytosed microparticles thus phenotypically and functionally resembled to antigen sampling M-like cells *in vivo*.

Initial work identified potential contribution of VTs to bacterium-host interactions and this investigation aimed to characterise roles of VT in colonization of bovine intestinal epithelium by EHEC. For this, adherence of a panel of wild-type and mutant (VT- negative) EHEC strains to primary rectal epithelial cells was assessed. Carriage of VT was associated with greater adherence to epithelium as demonstrated by higher capacity to form microcolonies compared to isogenic VT-negative strains. Pre-treatment of cells with VT produced a similar phenotype. VT exhibited further effects on epithelium through reduction of expression and secretion of IL-8, an important epithelial inflammatory mediator. VTs therefore do not show classic cytotoxicity for bovine intestinal epithelium but do exert pleiotropic effects on these cells, by modifying epithelial physiology hence enabling EHEC colonisation. The role of flagellae in interaction of *E. coli* O157:H7 with epithelium was also investigated. H7 flagellae acted as an adhesin only during the early stages of interaction as, at later stages, bacteria attached to actin-pedestals or in microcolonies did not express flagellae. Hence, H7 flagellum was identified as an adhesin, important in initial adherence of *E. coli* O157:H7 to bovine rectal epithelium.

Common Abbreviations

AB	alcian blue
AI	auto inducers
BSA	bovine serum albumin
EAST	ST-like enterotoxin
Efa 1	EHEC factor for adherence
EGF	epidermal growth factor
Epi	epinephrine
Esp	*E. coli* secreted protein
FAE	follicle associated epithelium
FCS	foetal calf serum
GALT	gut associated lymphoid tissue
HBSS	Hank's balanced salt solution
hly A	enterohaemolysin
HUS	haemolytic uraemic syndrome
Iha	irgA homologue adhesin
IrgA	iron regulated gene
LEE	locus of enterocyte effacement
ler	LEE encoded regulator
LF	lymphoid follicles
LPF	long polar fimbriae
MAP	mitochondrial associated protein
M-cells	membranous or microfold cells
Nle proteins	non-LEE encoded proteins
OmpA	outer membrane protein A
OsO_4	osmium tetraoxide
PAIs	pathogenecity islands
PAMPs	pathogen-associated molecular patterns
PAS	periodic acid schiff
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCM	pre-conditioned medium
PCNA	proliferating cell nuclear antigen
PET	plasmid-encoded heat-labile toxin
PFA	paraformaldehyde
PMNL	polymorphonuclear leukocytes
PRRs	pattern-recognition receptors
QS	quorum sensing
QseBC	quorum sensing *E. coli* regulator B and C
RAJ	recto-anal junction
RT	room temperature
Saa	STEC autoagglutinating adhesin
SPATE	serine protease autotransporters of *Enterobacteriaceae*
STEC	shiga-toxigenic *E. coli*
Stx	shiga-toxin
TccP	tir-cytoskeleton coupling protein
TLRs	toll-like receptors
TTP	thrombotic thrombocytopaenic purpura
TTSS	type III secretion system
VT	verotoxin
VTEC	verotoxigenic *E. coli*
ZSF	zinc acetate fixative

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1 Introduction

1.1 *Escherichia coli* (*E. coli*)

E. coli is a facultative anaerobic Gram negative bacillus, type species of the genus *Escherichia* that belongs to the family *Enterobacteriaceae*. Certain genera in this family are capable of causing a broad spectrum of intestinal and systemic diseases, e.g. *Salmonella typhi*, *Shigella dysenteriae* and *Yersinia pestis*. Many strains/serotypes of *E. coli* are predominantly found as commensal gut flora. However, in an immuno-compromised host these can cause severe enteric infections with systemic implications. *E. coli* is a species that has diverged into different serotypes/strain types with diverse virulence traits/determinants. These are encoded in clusters on virulence-related plasmids, chromosomal pathogenicity islands or on phages. *E. coli*, like any other mucosal pathogen, has evolved strategies to colonize the mucosal surface, evade or modulate host immune responses and secrete virulence factors that help in its colonization and persistence.

The species *E. coli* contains a diverse group of strains that have been classified on the basis of genetic relatedness and the type of patho-physiology of the disease these can cause: (i) urinary tract infections, (ii) sepsis/meningitis, and (iii) enteric infections/diarrhoea. The diarrhoeagenic strains have been classified further on the basis of their virulence factors and the disease pathology as described in the Table 1.1 including enterohaemorrhagic *E. coli* which forms the subject of this research.

1.1.1 Enterohaemorrhagic *E. coli* (EHEC)

The *E. coli* pathotype EHEC is closely related to enteropathogenic *E. coli* (EPEC) with the key difference that it produces Stx (shiga-toxin, also known as verotoxin/VT), and is commonly referred as shiga-toxigenic *E. coli* (STEC) or verotoxigenic *E. coli* (VTEC). Stx is an important virulence factor implicated in most of the serious disease sequelae in humans on infection with a STEC/VTEC. This toxin is encoded by a bacteriophage

Table1.1: Diarrhoeagenic *E. coli* (information compiled from (Nataro and Kaper, 1998).

Pathotype	Serotypes (examples)	Disease Characteristics	Major Toxins	Adherence Characteristics	Adhesins
Enterotoxigenic *E. coli* (ETEC)	O6:H16, O8:H9, O11:H27, O15:H11, O20:NM, O25:H42, O25:NM, O27:H7, O78:H11, O78:H12, O128:H7, O148:H28,O149:H10, O159:H20, O173:NM	Watery diarrhoea in young children and naive adults. Also host specific animal ETEC e.g. K99 in calves	Heat stable and/or heat labile toxins	Non-intimate diffuse adherence	Fimbriae (rigid rods, bundle-forming and fibrillar) and non-fimbrial adhesins
Enteropathogenic *E. coli* (EPEC)	O55:H6, O55:NM, O86:H34, O86:NM, O111:H2, O111:H12, O111:NM, O119:H6, O119:NM, O125ac:H21, O126:H27, O126:NM, O127:H6, O127:NM, O128:H2, O128:H12, O142:H6	Watery diarrhoea in infant humans and animals	Proteins secreted directly into host cells may affect function	Localised adherence, initially via fimbriae followed by intimate attachment	Bundle-forming pili and intimin/Tir interaction
Enterohaemorrhagic *E. coli* (EHEC)	O26:H11, O26:H32, O26:NM, O55:H7, O111ab:H8, O111AB:NM, O113:H21, O117:H14, O157:H7, O103:H2	Bloody diarrhoea that may progress to systemic disease syndromes	Shiga toxin, Enterohaemolysin, EAST1	As for EPEC	Intimin/Tir interaction and various putative adhesins
Enteroaggregative *E. coli* (EAEC)	O3:H2, O15:H18, O44:H18, O86:H18, O77:H18, O111:H21, O127:H2	Persistent, mucoid diarrhoea in developing countries	Enteroaggregative heat-stable toxin (EAST1),108 KDa Cytotoxin, plasmid-encoded heat-labile toxin (PET)	Aggregative adherence on HEp-2 cells and formation of bacterio-mucus biofilm *in vivo*	Aggregative adherence fimbriae I and II
Enteroinvasive *E. coli* (EIEC)	O28ac:NM, O29:NM, O112ac:NM, O124:H30, O124:NM, O136:NM, O136:NM, O143:NM, O144:NM, O152:NM, O159:H2, O159:NM, O164:NM, O164:NM, O167:H4, O167:H5, O167:NM	Outbreaks of watery diarrhoea	Possible enterotoxin	Invades with similar mechanism to *Shigella* spp.	pINV plasmid
Diffusely Adherent *E. coli* (DAEC)	O126:H27	Watery diarrhoea with higher incidence in older (4-5 years) children	None described	Non-intimate diffuse adherence	Fimbriae (F1845) AIDA-I

integrated into the chromosome of STEC (Scotland *et al.*, 1983). The strains of *E. coli* (STEC or VTEC) that cause disease in humans and possess the characteristic virulence determinants *viz* a virulence plasmid and LEE pathogenicity island (locus of enterocyte effacement) are classified as "enterohaemorrhagic *E. coli*" (EHEC) (Nataro and Kaper, 1998). Amongst different EHEC serotypes (Table 1.1), *E. coli* O157: H7 is the most widely-documented pathogen in human disease outbreaks.

EHEC are responsible for both sporadic and large-scale human outbreaks with symptoms of abdominal pain and non-bloody diarrhoea to haemorrhagic colitis (HC) marked by profuse quantities of blood in the faeces (Riley *et al.*, 1983). Some of the HC patients can go on to develop more severe systemic sequelae, most notably haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP). Stx is the cause of predominant pathology observed in these diseases (Karmali *et al.*, 1983). Classical HUS is characterised by the triad of microangiopathic haemolytic anaemia, thrombocytopaenia and acute renal dysfunction typically in young children. Acute renal failure leads to oligouria or anuria and haemolysis exacerbates the anaemia. TTP syndrome, frequently manifested by neurological disorders and fever, is confined to adults. TTP is usually not proceeded by a diarrhoeal phase (Pickering, Obrig, and Stapleton, 1994).

1.1.2 Animal reservoir of EHEC

Stx-producing *E. coli* have been found in faecal samples and meat products of a variety of farm animals and birds (Beutin *et al.*, 1993;Griffin, Olmstead, and Petras, 1990;Doyle and Schoeni, 1987). However, cattle are the principal reservoir hosts for *E. coli* O157:H7, the serotype most frequently associated with human infections. This is documented by various human outbreaks that can be traced back to consumption of undercooked beef and other bovine products such as unpasteurized milk (Borczyk *et al.*, 1987;Riley *et al.*, 1983) or drinking water (Yarze and Chase, 2000) (Licence *et al.*, 2001), vegetables and beverages (Swinbanks, 1996;Morgan *et al.*, 1988;Besser *et al.*, 1993) contaminated with cattle excreta. Humans can contract the infection directly from the cattle shedding the organism (Chapman, 2000) or indirectly. In addition to cattle, other domesticated and wild animals have also been identified as the source of infection in different outbreaks. Unpasteurized goat milk

(Bielaszewska *et al.*, 1997) and sheep dung (Ogden *et al.*, 2002) have been traced as a source of human outbreaks of *E. coli* O157:H7 infection. Pigs have been suggested to be a potential reservoir of *E. coli* O157:H7 (Nakazawa and Akiba, 1999). In wild habitats, white-tailed deer (*Odocoileus virginianus*) (Besser *et al.*, 1993) and rabbits (Pritchard *et al.*, 2001) have also been identified as possible carriers of *E. coli* O157:H7.

1.1.3 Prevalence of EHEC

Various epidemiological studies on farms and slaughterhouses have documented a worldwide presence of *E. coli* O157:H7 in cattle with prevalences of 1.8% in Japan (Miyao *et al.*, 1998), 1.9% in Australia (Cobbold and Desmarchelier, 2000), up to 1.8% in USA (Faith *et al.*, 1996), 8.6% in Scotland (Synge, 2000) and 4.7% in England and Wales (Paiba *et al.*, 2002). Many non-O157:H7 serotypes of EHEC have also been implicated with sporadic or large-scale human outbreaks. The most common non-O157:H7 serotypes associated with human disease include O26:H11, O103:H2, O111:NM, O113:H21 (Griffin, Olmstead, and Petras, 1990). The non-O157:H7 EHEC strains caused the majority of HUS cases in many countries like Chile (Ojeda *et al.*, 1995), Argentina (Lopez *et al.*, 1989), and Australia (Goldwater and Bettelheim, 2002) but worldwide O157:H7 is the most common.

1.2 Colonization and virulence factors of EHEC

Many factors are involved in the pathogenesis of EHEC including:

1.2.1 Shiga-Toxin (Stx)

Stx is the major virulence factor that plays a key role in pathogenesis of EHEC infections. It is reported to mediate (i) diarrhoea as a result of compromised intestinal epithelium integrity, (ii) haemorrhagic colitis due to damage to intestinal mucosal vasculature and (iii) damage to kidneys, circulatory and central nervous systems when disseminated systemically leading to life-threatening syndromes such as HUS and TTP (O'Brien and Holmes, 1987). The role of Stx in pathogenesis of these EHEC diseases has been modelled in various experimental studies (Matise *et al.*, 2001;Paton, Morona, and Paton, 2001).

1.2.1.1 Stx-Subgroups and Structure

Stx1 and Stx2 are two serologically-distinct subgroups of shiga toxins that may be expressed alone or together in an EHEC strain (Scotland *et al.*, 1983;Strockbine *et al.*, 1986). Stx1 is highly conserved and identical to Shiga toxin from *Shigella dysenteriae* whereas Stx2 has only 56% homology to Stx1 and has multiple variants designated as Stx2c, Stx2v, Stx2vhb, Stx2e etc. Stx is a compound holotoxin, comprising a single catalytic 32-kDa A subunit and pentameric B subunits (7.7-kDa monomers). A1 peptide (28-kDa) is the enzymatic component of the A subunit that binds to B subunit through the A2 peptide (4-kDa) (Sears and Kaper, 1996). The two subgroups of Stx vary in their toxicity and capacity to bind to certain human cell types (Jacewicz *et al.*, 1999).

1.2.1.2 Stx-Mode of action

Stx binds to eukaryotic cells via its pentameric B-subunit to a glycolipid receptor, globotriaosylceramide (Gb3 or CD77) (Lingwood, 1996).The bound toxin is endocytosed through clathrin-coated pits, transported within vesicles via the retrograde pathway to the Golgi apparatus and endoplasmic reticulum before being translocated to the cytosol (Sandvig *et al.*, 2002). During this process, subunit A is cleaved by the protease furin to release catalytically active A1 peptide which has N-glycosidase activity that removes a single adenine residue from the 28S subunit of 60S rRNA. The resultant inhibition of protein synthesis leads to apoptosis of sensitive cells like Vero or HeLa cells. However, in some cells the toxin bound vesicles undergo fusion with cellular endosomes/lysosomes, resulting in toxin degradation (Falguieres *et al.*, 2001;Hoey *et al.*, 2003b). Other additional cell cytotoxicity mechanisms have also been proposed for instance Stx-1 and Stx-2 induce apoptosis via inhibition of expression of anti-apoptotic Bcl-2 family members, Mcl-1 in endothelial cells (Erwert *et al.*, 2003). VT-1 also trigger apoptosis via activating caspase- and mitochondria-dependent pathways (Tetaud *et al.*, 2003).

1.2.1.3 Stx- Role in pathogenesis

Pathogenesis is a multi-step process involving a complex interaction of a range of bacterial and host factors. However, Stx is an important virulence factor essential for many of the pathological features as well as life-threatening sequelae of STEC

infections. Stx involvement in diarrhoea and enterocolitis have been documented in various animal model studies using Stx-mutated strains (Fontaine, Arondel, and Sansonetti, 1988) or adding bacteriophage encoding Stx1 to Stx-negative strains (Sjogren *et al.*, 1994). There is also evidence that Stx acts directly on the epithelium to induce pathological changes. Culture supernatants of *E. coli* O157:H7 and other EHEC serotypes provoked fluid accumulation in rabbit ileal loops, the severity of which correlated with the level of Stx (Ferreira *et al.*, 1997).

Stx interaction with intestinal epithelium is a key determinant in EHEC infection. Human intestinal epithelium lacks Gb3 (Holgersson, Jovall, and Breimer, 1991;Kasai *et al.*, 1985) and despite its absence, Stx is an important aetiological factor in mediating local and systemic effects. Stx can cross intestinal epithelium by a peri-cellular route (Acheson *et al.*, 1996;Hurley *et al.*, 1999;Philpott *et al.*, 1997;Lauvrak, Torgersen, and Sandvig, 2004) and localise to sub-epithelial tissues. There it may cause local damage to Gb3-expressing endothelium of the mucosal vasculature as observed in haemorrhagic colitis. Another crucial role of Stx is induction of pro-inflammatory mediators including TNF-α and IL-6 (Harel *et al.*, 1993;Tesh, Ramegowda, and Samuel, 1994), IL-1β and IL-8 by epithelia, which damage the intestinal mucosa directly by inflammation or indirectly by recruitment of phagocytic cells (Thorpe *et al.*, 1999;Yamasaki *et al.*, 1999). TNF-β and IL -1β can enhance Stx cytotoxity by increasing the expression of Gb3 and hence toxin binding to human endothelial cells (Kaye *et al.*, 1993;Louise and Obrig, 1991;van de Kar *et al.*, 1992). The Gb3-positive polymorphonuclear leukocytes (PMNL) (Ramegowda and Tesh, 1996;Foster and Tesh, 2002;Molostvov *et al.*, 2001;Yoshida *et al.*, 1999;Sears and Kaper, 1996) localised at the EHEC colonised site sequester the Stx and disseminate it systemically in a "Trojan horse" mechanism leading to HUS or TTP (Hurley, Thorpe, and Acheson, 2001;Te Loo *et al.*, 2000b).

1.2.1.4 Stx in cattle

Cattle are an asymptomatic carrier host for EHEC strains particularly *E. coli* O157:H7 (Chapman *et al.*, 1993;Synge, 2000). Although Stx has been detected to biologically-relevant levels in faeces of cattle (Ball *et al.*, 1994) (Hyatt, Galland, and Gillespie, 2001), there is no evidence that it is toxic to the ruminant host. One

possible explanation for the lack of virulence in cattle may be that the Gb3-positive basal crypt cells in the intestinal epithelium sequester the Stx and processes it to lysosomes where it is functionally neutralized (Hoey *et al.*, 2003a). In addition, the distribution and isotype of Gb3 receptors in bovine organs may account for lack of virulence in this host. Nevertheless, presence of anti-Stx antibodies in the sera and colostrum of cows (Johnson, Cray, Jr., and Johnson, 1996;Pirro *et al.*, 1995) provides evidence that Stx is recognized as an antigen and is capable of evoking immune responses (Menge *et al.*, 2004).

Stx is a potent immuno-stimulatory molecule as evidenced in previous studies using mouse models (Harel *et al.*, 1993;Tesh, Ramegowda, and Samuel, 1994). However in cattle, Stx has been shown to be immunosuppressive. Stx reduced the mitogen-induced proliferation and activation of bovine peripheral blood mononuclear cells (PBMC) without inducing apoptosis (Menge *et al.*, 1999). In cultures of bovine intraepithelial lymphocytes, Stx down-regulated the secretion of proinflammatory cytokines including IL-8 (Menge *et al.*, 2004), a strong chemoattractant for neutrophils. Hence, Stx may limit local tissue damage and might help in innocuous colonisation and persistence of EHEC organisms in the cattle gut.

1.2.2 Locus for Enterocyte Effacement (LEE)

LEE is a pathogenicity island that codes for many virulence factors important in the pathogenesis of EHEC infection.

1.2.2.1 LEE organisation

The virulence genes necessary for the characteristic histopathology of A/E (attaching/ effacing) lesions associated with EHEC and EPEC colonisation are interspersed in a large chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE). This is a 35.5 kb cluster of genes comprising over 40 open reading frames (ORFs) organised into several polycistronic operons that determine the ability to form intimate attachment and the associated attaching and effacing lesion on the apical surface of the host cells (Fig. 1.1). The LEE pathogenicity island is commonly inserted within the *selC* tRNA locus in EHEC and EPEC (Wieler *et al.*, 1997). Although *E. coli* O157:H7 is considered to have evolved

from an EPEC O55 strain (Reid *et al.*, 2000), the LEE of EPEC but not of EHEC confers the A/E phenotype on *E. coli* K12 suggesting a divergence of LEE proteins or regulation between these two pathotypes (McDaniel and Kaper, 1997;Elliott, Yu, and Kaper, 1999). The O157:H7 LEE differs from EPEC locus by the insertion of a putative prophage that codes for 13 more ORFs (Perna *et al.*, 2001).

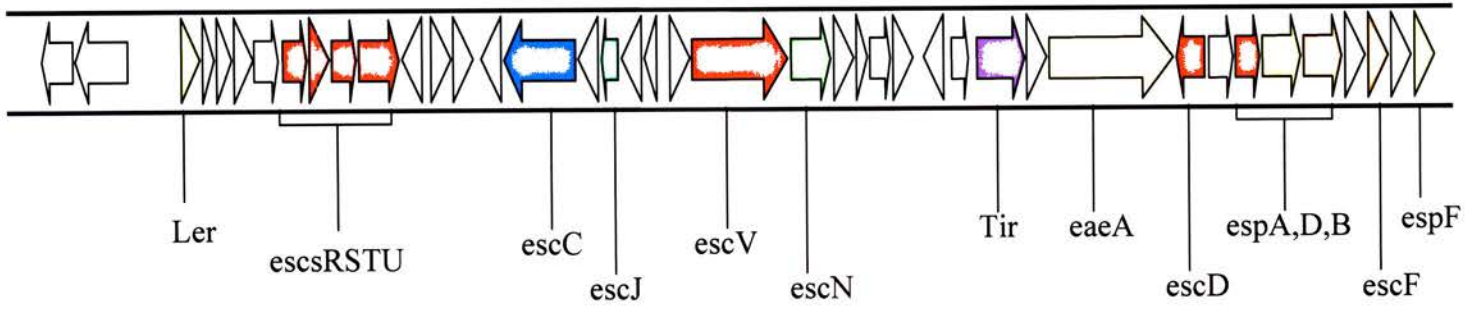

Fig. 1.1 Arrangement of genes on LEE pathogenicity island of *E. coli* O157:H7

The LEE genes determining the A/E phenotype are arranged in five operons (LEE1 to 5) of which the first three (LEE1, 2, 3) are identical between EPEC O127 and EHEC O157, and code for a type III secretory apparatus (Perna *et al.*, 1998;Elliott *et al.*, 1998;Kaper, 1998). This is a contact-dependent mechanism in which proteins are secreted across bacterial and host cell membranes and inserted directly into the host cell cytoplasm (Hueck, 1998). LEE4 encodes secreted proteins EspA, B and D which form the translocon and a pore required to deliver effector proteins into the cytoplasm of the host cell (Ebel *et al.*, 1998a;Delahay *et al.*, 1999;Knutton *et al.*, 1998;Kresse, Rohde, and Guzman, 1999;Shaw *et al.*, 2002;Wachter *et al.*, 1999;Ide *et al.*, 2001;Tacket *et al.*, 2000). LEE5 encodes *eae* and *tir* genes, the former encodes an outer membrane protein, intimin, that binds to a receptor Tir (translocated intimin receptor), inserted into the host cell. Other smaller operons and ORFs are also present in the LEE that code for various secreted/effector proteins including EspF (Crane, McNamara, and Donnenberg, 2001), EspG (Elliott *et al.*, 2001), EspH (Tu *et al.*, 2003), MAP (mitochondrial associated protein) (Kenny and Jepson, 2000), Tir (Kenny *et al.*, 1997) and a chaperone protein CesT for Tir (Abe *et al.*, 1999). Many of these effector proteins are involved in modulating the host cytoskeleton.

1.2.2.2 Intimin and Tir

Intimin is the adhesin essential for intimate attachment of EHEC and the subsequent formation of A/E lesions. Bacteria intimately attach to host cells using this 94- to 97-kDa outer membrane protein and its receptor Tir, injected via Type III secretion into the host cell (Knutton *et al.*, 1998;Kenny *et al.*, 1997;Jerse *et al.*, 1990). Studies with *eaeA*-negative O157:H7 mutants have shown that intimin is essential for A/E lesion formation both in cultured cells (Bower *et al.*, 1989;Fang *et al.*, 1995;Cookson and Woodward, 2003) and experimental animal models using gnotobiotic piglets (McKee and O'Brien, 1995;Tzipori *et al.*, 1995), colostrum-deprived calves (Dean-Nystrom *et al.*, 1998) and sheep (Woodward *et al.*, 2003). It is also required for persistence in experimentally-challenged weaned calves (Cornick, Booher, and Moon, 2002b). However intimin-negative EHEC serotype O113:H21 has also been isolated from HUS patients. This serotype expressed a distinct adhesion phenotype with areas of microvillus effacement in the absence of actin polymerization on cultured epithelial

cells (Hep-2) and rabbit intestine *in vivo* (Dytoc *et al.*, 1994). The structural basis of intimin-Tir interaction is well defined. The Tir-binding domain of intimin consists of the C-terminal 280 amino acids (Int280) that includes a C-type lectin-like domain and a part of an immunoglobulin domain (Batchelor *et al.*, 2000;Kelly *et al.*, 1999;Luo *et al.*, 2000), while the intimin-binding domain of Tir consists of 109 amino acids comprising of 2 helices joined by an α hairpin-turn (de Grado *et al.*, 1999).

Although Tir is considered as the primary receptor for intimin, Tir-independent binding to other host cell receptors has been reported. In Hep-2 cells, EHEC O157 intimin was shown to co-localize with nucleolin and the binding was inhibited by anti-nucleolin polyclonal sera, confirming nucleolin as a putative receptor for intimin on the surface of these cells (Sinclair and O'Brien, 2002;Sinclair and O'Brien, 2004). A Cys937Ala substitution in intimin that abolished the formation of 76-amino acid loop had no effect on intimin-Tir binding but was essential for intimin binding to Hep-2 cells and *in vivo* models of pathogenicity (Hartland *et al.*, 1999). The carboxy terminal of intimin mediated attachment to, and remodelling of the surface of Hep-2 cells in absence of Tir (Phillips *et al.*, 2000a). However, there is no evidence of Tir-independent interaction(s) of intimin in colonisation of *E. coli* O157:H7 in the ruminant host. In a recent study, the mutation of *tir* caused a significant reduction in faecal shedding of *E. coli* O157:H7 in calves indicating the importance of Tir in colonisation of the bovine intestine (Stevens *et al.*, 2004a).

Intimin proteins of EHEC and EPEC strains show high conservation in the N-terminal region and variability in the last 280 C-terminal amino acids of the protein. The antigenic polymorphism at the C-terminal region, where binding to enterocytes and Tir occurs, has led to the classification of intimins into at least 10 different types (Table 1.2) (Adu-Bobie *et al.*, 1998a;Jores *et al.*, 2003;Oswald *et al.*, 2000). The initial studies using genetic and immunological approaches provided evidence for the existence of at least four distinct intimin types known as intimin α, ß, γ, and δ (Adu-Bobie *et al.*, 1998a;Agin and Wolf, 1997). A subsequent study revealed the presence of a fifth type, intimin ε, in *E. coli* strains of serogroup O103 (Oswald *et al.*, 2000).

It has also been proposed that intimins α, ß, and γ can be further subdivided based on restriction digestion analysis into α1,α2, ß1, ß2, γ1 and γ2, with ß2 being identical to δ (Oswald *et al.*, 2000). Recently, three independent studies have proposed that additional intimin types based only on subtle differences at the nucleotide level can be found in other EPEC or EHEC strains. These new groups have been classified as intimins ζ, η, θ, ί, and κ (Jores *et al.*, 2003) (Tarr and Whittam, 2002;Zhang *et al.*, 2002).

Table 1.2: Different Intimin types and their tissue tropism (adapted from (Zhou *et al.*, 2003b).

Intimin type	*E. coli* serotype (examples)	Host and/ or tissue specificity	Reference
α (alpha)	EPEC clone 1 O127:H6 and O114:H2	Human proximal and distal small intestines	(Adu-Bobie *et al.*, 1998b;Phillips and Frankel, 2000)
β (beta)	EPEC clone 2, EHEC clone 2, RDEC-1	Animal and human (Peyer's patch lymphoid follicles in rabbits)	(Adu-Bobie *et al.*, 1998b;Cantey and Inman, 1981)
γ (gamma)	EHEC O157:H7, EPEC O55:H7 and O55:H$^-$	Human ileal FAE	(Fitzhenry *et al.*, 2002a;Phillips and Frankel, 2000)
δ (delta)	EPEC O86:H34	Human	
ε(epsilon)	EHEC O8, O11, O45, O113, O121, and O165	Bovine and human (O113 strains adhere to large intestine FAE)	(Oswald *et al.*, 2000;Fitzhenry *et al.*, 2003)
ζ (zeta)	O84:NM	Bovine	(Jores *et al.*, 2003)
η (eta)	O125: H$^-$, O84:H2	Human	(Zhang *et al.*, 2002)
θ (theta)	O111:H8	Human	(Zhang *et al.*, 2002)
ί (iota)	O145:H4, O98:H2	Human	
κ (kappa)	O118:H5	Human	(Zhang *et al.*, 2002)

The type of intimin correlates to the pattern of colonisation and tissue tropism in different hosts. Intimin α is expressed by EPEC clone 1 and seems to confer specificity for the Peyer's patches as well as human proximal and distal small intestines (Phillips and Frankel, 2000); intimin ß is associated with human and animal EPEC 2 and EHEC 2 strains and is expressed in rabbit Peyer's patch lymphoid follicles (Cantey and Inman, 1981); intimin γ expression correlates with colonization of human ileal Peyer's patches by EHEC O157:H7, EPEC O55:H7, and EPEC O55:H$^-$(Fitzhenry *et al.*, 2002a;Phillips and Frankel, 2000). Although intimin ε is expressed by human and animal EHEC strains other than those of serogroup O157, the tissue distribution of these strains is similar to that observed with EHEC O157:H7 strains expressing intimin γ (Fitzhenry *et al.*, 2003).

The host tissue distribution of EPEC and EHEC strains is probably multifactorial, but different intimin types may be crucial to tissue- and host- specific tropism. Intimin exchange studies performed in piglets and human intestinal explants showed that, while EPEC expressing intimin α colonised Peyer's patches as well as proximal and distal regions of small intestine, expression of EHEC intimin γ in the EPEC background conferred tissue tropism restricted to Peyer's patches only (Phillips and Frankel, 2000). Studies of bovine attaching and effacing *E. coli* isolates demonstrated a different distribution of intimin types in strains associated with diarrhoeic calves compared to healthy adults. Strains isolated from adults were more likely to possess intimin γ, whereas diarrhoeic calf isolates mostly possessed intimin β. However this variation in pathotype may not have been mediated by intimin type alone since different Tir, EspA and EspB sub-types are associated with certain intimin types (China *et al.*, 1999a;China *et al.*, 1999b) in addition to other serotype-dependent differences. Reports of calf diarrhoea, both natural and experimental, where A/E lesions have been demonstrated, invariably involve strains with non-γ intimin types such as β (O26) (Pearson *et al.*, 1999), O80 (Wray, McLaren, and Pearson, 1989) and O118 (Stordeur *et al.*, 2000), whereas *E. coli* O157:H7 has only been reported to cause disease in experimentally infected neonatal calves (Dean-Nystrom, Bosworth, and Moon, 1999).

1.2.2.3 A/E lesion formation

A/E lesion formation is complex and triggered by a number of host and bacterial proteins acting in unison. On initial attachment to host epithelial cells, EHEC and EPEC elongate a translocon apparatus (consisting of EspA as the main structural protein and the membrane pore forming proteins EspB & EspD) to the host cell, transfer effector proteins through the translocon and form an intimate attachment via intimin/Tir interaction. This is followed by formation of a micro-colony (MC) on the cell surface, condensation of F-actin underneath the MC, leading to effacement of microvilli and formation of pedestal-like structures.

The bacterial effector proteins including Tir, EspF and MAP, inserted into the host cell are capable of subverting various cellular processes, including cytoskeletal

rearrangements, apoptosis and cytokine release. Tir is a multi-functional protein which, apart from being a receptor for intimin, initiates pedestal formation. Following intimin/Tir binding, Tir undergoes phosphorylation within the C-terminal 170 amino acids and serves as a nucleator of actin polymerisation. It directs the recruitment of several cytoskeletal proteins to the site of EHEC attachment. These include α-actinin, ezrin, cortactin, talin, fimbrin, vasodilator-stimulated protein (VASP), neural Wiskott-Aldrich syndrome protein (N-WASP) and actin-related protein (Arp) 2/3 complex (Goosney, DeVinney, and Finlay, 2001a;Ismaili *et al.*, 1995;Cantarelli *et al.*, 2002). In EHEC the recruitment is independent of Tir phosphorylation yet the composition of the pedestal is similar to EPEC with the difference of adaptor proteins, growth-factor-receptor-bound protein 2 (Grb2) and CrkII. Grb2 is an activator of N-WASP and the Arp2/3 complex (Carlier *et al.*, 2000), both of which are essential in pedestal formation in EPEC (Kalman *et al.*, 1999). Differences in the modification of Tir in EHEC have led some workers to suggest that either EHEC delivers its own adaptor proteins into the host cytoplasm to initiate actin nucleation or that Tir phosphorylation is not essential for this process (DeVinney *et al.*, 2001). Recently, a novel EHEC effector protein TccP (Tir-cytoskeleton coupling protein) that displays activity similar to host adaptor protein Nck, has been identified to play a role in recruitment of cytoskeletal proteins α–actinin, Arp3, N-WASP and actin to the site of bacterial adhesion (Garmendia *et al.*, 2004). This proline-rich protein, $EspF_U$ resembles EspF and is the only EHEC effector of actin assembly absent from EPEC (Campellone, Robbins, and Leong, 2004).

1.3 Other virulence factors of EHEC

1.3.1 EHEC 60-MDa plasmid

Most of the Stx-producing strains isolated from humans possess plasmid(s) (Beutin *et al.*, 1994;Levine *et al.*, 1987). All strains of O157 contain a highly-conserved 60MDa plasmid (Schmidt, Karch, and Beutin, 1994), which has been documented to

enhance adhesion to intestinal epithelium when tested in the *E. coli* K12 background in a rabbit challenge experiment (Dytoc *et al.*, 1993). A plasmid-cured derivative of *E. coli* O157:H7 had a reduced adherence to cultured cells (Toth *et al.*, 1990) but had no effect on disease pathology in rabbit (Li *et al.*, 1993) and piglet (Tzipori *et al.*, 1987) models of disease. Earlier it was believed that this plasmid encoded fimbrial genes (Karch *et al.*, 1987), however, sequencing of pO157 revealed no such fimbrial operons (Burland *et al.*, 1998). pO157 contains several putative virulence genes, such as *hlyA* (enterohaemolysin) (Schmidt, Beutin, and Karch, 1995), *esp*P encoding a SPATE (Serine Protease Autotransporter Toxin of Enterobactericaeae) toxin (Brunder, Schmidt, and Karch, 1997) and *toxB* which contains some motifs homologous to *Clostridium difficile* toxins A and B and *efa-1*. ToxB has been reported to contribute to full expression of type III secreted proteins required for *E. coli* O157:H7 adherence to host cells (Tatsuno *et al.*, 2001;Stevens *et al.*, 2004a). *saa* (STEC autoagglutinating adhesin) is another novel plasmid-encoded virulence gene associated with *ehxA* genes that has been detected in LEE-negative strains implicated with HUS (Paton *et al.*, 2001;Paton and Paton, 1999). In a survey the *saa* gene was found associated significantly with bovine STEC strains suggesting that it may have a role in colonization of bovine gut (Jenkins *et al.*, 2003a).

1.3.1.1 Enterohaemolysin (Ehx)

Ehx belongs to the RTX toxin family, members of which are expressed by uropathogenic *E. coli*, *Pasteurella haemolytica*, and other human and animal pathogens (Bauer and Welch, 1996). EHEC strains expressing Ehx produce weak zones of haemolysis on sheep blood agar containing washed erythrocytes (supplemented with Ca^{2+}) after 18 to 24 hours (Beutin *et al.*, 1989). Ehx is highly conserved amongst STEC strains suggesting that it contributes to survival in someway (Boerlin *et al.*, 1999b), however, there is conflicting evidence regarding its contribution to the development of HC or HUS (Schmidt, Kernbach, and Karch, 1996;Boerlin *et al.*, 1999b). Ehx is produced at very low levels by *E. coli* O157:H7 and this is thought to be due to a defective transporter system (Schmidt, Beutin, and Karch, 1995). However, the expression of Ehx has been shown to be optimal *in vitro* under conditions of low oxygen tension (Chart *et al.*, 1998). Expression of the structural toxin gene *in vivo* has been demonstrated indirectly through the detection

of Ehx-specific antibody in bovine colostra (Lissner, Schmidit, and Karch, 1996) and sera of HUS patients (Schmidt, Beutin, and Karch, 1995).

1.3.1.2 Serine protease (EspP)

The *Esp* (*E. coli* secreted protein P) gene of *E. coli* O157:H7 is a member of the SPATE family of toxins and is one of the most abundant proteins secreted during growth in LB medium (Brunder, Schmidt, and Karch, 1997). EspP has been reported to be cytotoxic for Vero cells. The protease cleaved human coagulation factor V and hence could result in exacerbation of haemorrhagic disease in STEC- infected patients. A role of EspP in pathogenesis is consistent with presence of antibodies to the protease in sera from STEC infected patients (Brunder, Schmidt, and Karch, 1997).

1.3.1.3 StcE

StcE (secreted protease of C1 inhibitor from EHEC), a metalloprotease, is secreted by *etp* type II secretion pathway encoded on pO157, and the extracellular levels of StcE are positively regulated by the LEE-encoded regulator, Ler. This is a member of the serine protease inhibitor family that specifically cleaves C1 esterase inhibitor (C1-INH). In the presence of StcE, cultured human T-cells (Jurkat cells) formed aggregates but neither proliferated nor produced IL-2. Presence of StcE antigen in the faeces of an *E. coli* O157:H7 infected child correlates with the biological activity attributed to this protein (Lathem *et al.*, 2002).

1.3.1.4 ToxB

The sequence of the *toxB* gene shows N-terminal homology to the ToxA and ToxB proteins of *C. difficile* and contains a conserved region essential for glycosyltransferase activity in the clostridial toxins (Burland *et al.*, 1998). Tatsuno *et al.*, obsereved that deletion of pO157 (O157Cu) reduced adherence to Caco-2 cells and on introduction of a mini-pO157 plasmid (pIC37) composed of the *toxB* and ori regions full adherence capacity was restored (Tatsuno *et al.*, 2001). However, an indirect mechanism for this activity was proposed by the observation that a mutation in *toxB* leads to a four-fold decrease in *LEE*4 protein (EspA, B and D) secretion levels. A *toxB* homologue is present in non-O157 EHEC strains such as *E. coli* O111, termed Efa 1 (EHEC factor for adherence), that appears to contribute to initial

adherence of the organism to CHO cells (Nicholls, Grant, and Robins-Browne, 2000). It has been identified as an essential factor in experimental colonisation of cattle by *E coli* O111 and O5 (Stevens *et al.*, 2002b), although this may be an indirect consequence of its possible role in the *LEE* type III secretion system (Tatsuno *et al.*, 2001). The EHEC O111:H- Efa1 protein is 97.4% identical to EPEC lymphostatin (LifA), which confers the ability to inhibit lymphocyte proliferation of human peripheral blood lymphocytes and the mitogen- activated synthesis of proinflammatory cytokines (Klapproth *et al.*, 2000). LifA also inhibits the proliferation of human and murine intraepithelial lymphocytes, indicating that LifA and Efa1 may influence intestinal colonisation by modulating mucosal immunity in the gut (Klapproth *et al.*, 1995;Klapproth *et al.*, 2000). However, recently it was shown that, despite affecting type III secretion, mutation of *toxB* and *efa-1* did not significantly affect the course of faecal shedding of *E. coli* O157:H7 following experimental inoculation in calves or sheep (Stevens *et al.*, 2004a).

1.3.2 Fimbrial Adhesins of EHEC

The complete genome sequence of *E. coli* O157:H7 EDL 933 has defined the presence of various putative fimbrial and non-fimbrial adhesin gene clusters that are unique to O157 and likely to be important in colonisation of the host (Perna *et al.*, 2001). Of the fourteen putative fimbrial operons, four are common to *E. coli* K12 MG1655 sequence which includes the type 1 fimbrial operon. The majority of the *E. coli* O157:H7 isolates possess the type 1 fimbrial operon (Enami *et al.*, 1999), but are not able to express it due to a conserved deletion in its promoter region (Roe *et al.*, 2001). In a *ler*-mutated EHEC strain, presence of long fine fimbriae were observed to enhance adherence to cultured cells (Elliott *et al.*, 2000b) however these fimbrial structures were not specifically identified at that time.

Two fimbrial operons closely related to long polar (LP) fimbriae of *Salmonella enterica* serovar *Typhimurium* have been identified in *E. coli* O157:H7. Over-expression of LP fimbrial operon 1 *lpfABCCĆDE* in non-fimbriated *E. coli* K12 promoted microcolony formation but did not enhance adherence to a cultured cell line (Torres *et al.*, 2002a). LP fimbrial operon 2 has recently been characterised in EHEC O113:H21. Mutation of this locus in O113:H21 resulted in decreased

adherence to epithelial cells suggesting that lpf (O113) may function as an adhesin in LEE-negative isolates of EHEC (Doughty *et al.*, 2002). In a recent experimental study in sheep and pigs, the *lpf1* and *lpf2* mutants of *E. coli* O157:H7 were recovered in significantly lower numbers and caused fewer A/E lesions than the parent strain (Jordan *et al.*, 2004). In another study, neither *lpf1* nor *lpf2* mutants showed an effect on adherence or A/E lesion formation during *in vitro* studies on cultured cell lines although since *lpf2* mutants were deficient in adherence to Caco-2 cells at early time points, their role in early stages of infection was indicated (Torres *et al.*, 2004).

Curli fibres are thin aggregative fimbriae involved in bacterial adhesion to host proteins and in biofilm formation. The curli fibres are infrequently expressed during *in vitro* growth of *E. coli* O157:H7 in a temperature-independent phase-variant manner (Uhlich, Keen, and Elder, 2001). Strains containing variations at the *csgD* promoter region, which induced expression of curli, are associated with increased virulence in mice and increased invasion of cultured epithelial cells (HEp-2) (Uhlich, Keen, and Elder, 2002). The curli subunit protein (CsgA) of *E. coli* O157:H7 contains an extra glycine and is structurally distinct from K-12 curli fibres. Transposon mutagenesis studies in *E. coli* O157:H7 identified a hyperadherent mutant phenotype with disrupted *csgD*, thus the *csgD* product may be controlling the expression of an adhesive factor required for binding to HeLa cells (Torres and Kaper, 2003).

1.3.3 Non-fimbrial adhesins of EHEC

Iha (IrgA homologue adhesin), a 67 kDa outer membrane protein, identified by random mutagenesis was found to confer adherence phenotype to *E. coli* O157:H7 when tested on cultured epithelial cells (Tarr *et al.*, 2000). It is encoded by a chromosomally located *Iha* gene similar to iron-regulated gene (IrgA) of *Vibrio cholerae* (Goldberg *et al.*, 1992). OmpA (Outer Membrane Protein A) was associated with a hyperadherent phenotype of *E. coli* O157:H7 and its role as adhesin was indicated. Disruption of *ompA* gene or treatment with anti-OmpA serum abolished the hyperadherent phenotype of this transposon insertion mutant (Torres and Kaper, 2003). As noted above, Efa1 was found necessary in colonization by STEC serotypes O5 and O111 in calf challenge experiments (Stevens *et al.*, 2002b). Earlier Nicholls

et al., observed an essential role of *efa*1 in adherence of STEC O111:H- to CHO cells (Nicholls, Grant, and Robins-Browne, 2000). A truncated version of *efa1* exists in the chromosome of *E. coli* O157:H7 and a transposon insertion mutation upstream of *efa1* reduced the adherence to Caco-2 cells (Tatsuno *et al.*, 2000).

E. coli O157:H7 strains deficient in LPS O-antigen side chains displayed an increased binding to cultured epithelial cells (Bilge *et al.*, 1996;Cockerill, III *et al.*, 1996;Torres and Kaper, 2003;Tatsuno *et al.*, 2003). Two copies of a gene (*cah*) that encode a calcium-binding autotransporter protein may play a role in biofilm formation. Expression of Cah in *E. coli* DH5α increased bacteria-to-bacteria interactions but had no role as an adhesin in binding assays on HeLa cells (Torres *et al.*, 2002b) and therefore it is possible that this may play a role in microcolony formation. Two proteins, YhiE and YhiF, negatively regulated the expression of LEE genes and hence affected the adherence of O157Sakai to Caco-2 cells. During experimental studies in mice the *yhiE* mutant O157Sakai colonized better than the wild type strain (Tatsuno *et al.*, 2003).

1.3.4 Non-LEE encoded (Nle) effector proteins

Many key virulence factors shared by A/E pathogens are encoded in the LEE pathogenicity island essential for A/E lesion formation. The LEE contains 41 genes that encode a type III secretion system (TTSS) that delivers into the host cell bacterial effector proteins capable of modulating host functions. Five LEE-encoded effector proteins (Tir, EspG, EspF, Map and EspH) have been identified which are involved in modulating host cytoskeleton. Recently seven effectors proteins encoded by pathogenicity islands outside the LEE have been identified to be secreted by TTSS in *Citrobacter rodentium*, six of which are highly conserved in EHEC and EPEC (Deng *et al.*, 2004). In EHEC these effectors (NleA, NleB, NleC, NleD, NleE and NleF) are encoded outside the LEE by three PAIs (O-islands 36, 71, and 122) that are present in many A/E pathogens (Perna *et al.*, 2001;Morabito *et al.*, 2003). NleA, a protein of approximately 50 kDa, secreted via TTSS into the host cell localizes to the Golgi apparatus. Although NleA does not affect adherence of bacteria, it can prime the host immune system and initiate a protective immune response (Gruenheid *et al.*, 2004).

1.3.5 H7 flagellae

The role of H7 flagellae in the biology of *E. coli* O157:H7 infections is obscure. Studies by Sherman and Soni (Sherman and Soni, 1988) showed that antibodies to whole cells or outer membranes, but not to H7 flagellae, significantly inhibited the adherence of O157:H7 STEC to Hep-2 cells. Moreover, exogenous addition of OMP extracts inhibited adhesion in a concentration-dependent manner whereas addition of isolated flagellae and LPS did not. Giron *et al.* demonstrated a role for flagellae in EPEC adherence. They showed that a *fliC* mutant, that has an insertion in the gene encoding flagellin, exhibited 60 percent reduction in adherence. Purified H6 and H2 flagellae bound to HeLa cells as observed by immunofluorescence and anti-H6 and anti-H2 antibodies inhibited bacterial adherence. It was also demonstrated that *E. coli* O157 (EDL933) in microcolonies neither expressed flagellae nor did the purified H7 flagellae adhere to HeLa cells and hence the role of H7 flagellae in adherence of *E. coli* O157 (EDL933) was ruled out (Giron *et al.*, 2002).

1.4 Flagellae

The flagellum is a multifaceted organelle that plays many distinctive roles in the biology of a microbe. As a motility organelle, it confers distinct advantages upon host-adapted prokaryotes. Potential benefits of motility include increased efficiency of nutrient acquisition, avoidance of toxic substances, ability to translocate to preferred hosts and access optimal colonization sites within them, and dispersal in the environment during the course of transmission. A growing number of investigations have incriminated flagellae in virulence (for example adherence, invasion and proinflammatory responses) in several bacterial pathogens (Table. 1.3).

Table 1.3: The role of flagellae in virulence of selected bacterial pathogens.

Bacteria	Flagellae mediated role in virulence	Reference
Aeromonas caviae	Adherence and invasion of Hep-2 cells	(Rabaan *et al.*, 2001)
Salmonella enterica serovars Typhimurium and Enteritidis	Persistence and inflammatory responses in the upper gastrointestinal tract of rat; Penetration of gastrointestinal mucus layer and attachment to epithelial cells in rat ilial explants; adherence and colonization in chicken model of infection	(Robertson *et al.*, 2003;Robertson *et al.*, 2000;Dibb-Fuller *et al.*, 1999;Allen-Vercoe and Woodward, 1999)
Avian pathogenic *E. coli* strains (*E. coli* O78:K8)	Adherence to mucus-secreting cell line HT2916E, Colonization and persistence in chicken model of infection	(La Ragione, Cooley, and Woodward, 2000;La Ragione, Sayers, and Woodward, 2000)
Helicobacter pylori	Colonization and persistence of gnotobiotic piglets	(Eaton *et al.*, 1996;Eaton, Morgan, and Krakowka, 1992)
Proteus mirabilis	Colonization of urinary tract in mice model of infection	(Mobley *et al.*, 1996a)
Yersinia enterocolitica	Invasión of HEp-2 cells	(Young, Badger, and Miller, 2000)
Pseudomonas aeruginosa	Biofilm formation; Adherence to MUC1 mucin; asialoGM1 (ASGM1); induction of matrilysin expression in cystic fibrosis, phosphorylation of extracellular signal-regulated kinase (ERK)	(O'Toole and Kolter, 1998;Lillehoj, Kim, and Kim, 2002);(McNamara *et al.*, 2001a); (Lopez-Boado, Wilson, and Parks, 2001); (Lillehoj *et al.*, 2004)
Vibrio cholerae	Biofilm formation	(Watnick *et al.*, 2001)
Clostridium difficile	Adherence to mouse cecal mucus	(Tasteyre *et al.*, 2001)
Burkholderia pseudomallei	Entry in to *Acanthamoeba astronyxis* trophozoites	(Inglis *et al.*, 2003)
Aeromonas spp.	Biofilm formation and adherence to intestinal cell lines :Henle 407 and Caco-2	(Kirov, Castrisios, and Shaw, 2004b)
Listeria monocytogenes	Adherence and invasion of Caco-2 cells	(Dons *et al.*, 2004)
E. coli O127:H6 (E2348/69), *E. coli* O119:H6, *E. coli* O114:H2	Adherence and microcolony formation on HeLa cells	(Giron *et al.*, 2002)

1.4.1 Flagellaer gene system

The genes regulating flagellae expression and biogenesis are divided among 17 operons, constituting a large and co-ordinately regulated flagellaer regulon. Within the regulon, the operons are divided into three temporally-regulated, hierarchial transcriptional classes: class 1, class 2 and class 3 (Kutsukake, Ohya, and Iino, 1990). The class 1 genes encode the master flagellae regulator FlhDC, which activates the transcription of class 2 genes, including genes encoding for the structure and assembly of the hook-basal body and the transcriptional regulators FlgM and FliA (σ^{28}). FliA regulates the transcription of class 3 genes, which include *fliC* (flagellin) and the *mot* operon. *flhDC* expression is regulated in response to several environmental cues (reviewed by Chilcott and Hughes, 2000) including bacterial cell

density (Sperandio, Li, and Kaper, 2002;Sperandio, Torres, and Kaper, 2002;Sperandio *et al.*, 2001) and host-secreted factors (Sperandio *et al.*, 2003).

1.4.2 Cross-talk between flagellae and LEE-genes in EHEC

The flagellum consists of components that function as a type III secretory system (TTSS), facilitating the export of flagellar proteins and flagellar assembly (Aldridge and Hughes, 2002). EPEC and EHEC contain an additional TTSS encoded by several operons located in the LEE pathogenicity island (McDaniel and Kaper, 1997). Most of the LEE operons (LEE2, LEE3, LEE4, LEE5) are positively regulated by the Ler regulator, which is encoded by the *LEE1* operon (Friedberg *et al.*, 1999;Mellies *et al.*, 1999;Sperandio *et al.*, 2000;Sanchez-Sanmartin *et al.*, 2001). The LEE-encoded TTSS and flagellar biogenesis in EPEC and EHEC are regulated in a mutually exclusive fashion by integration host factor (IHF), that directly activates the expression of Ler (Friedberg *et al.*, 1999) and represses the flagellar expression by silencing *flhDC* regulon (Yona-Nadler *et al.*, 2003). The cross talk between LEE-operons and flagellar biogenesis in EHEC is also regulated by quorum sensing.

Quorum sensing (QS) is a cell-to-cell signalling mechanism in which bacteria secrete hormone-like compounds called autoinducers (AI). When these AIs reach a certain threshold concentration, they interact with bacterial transcriptional regulators, and thereby regulate gene expression. In EHEC, the LEE-genes encoding TTSS are regulated by QS through AI-2 (Sperandio *et al.*, 1999). The AI-2 is a by-product of metabolic enzyme LuxS, involved primarily in the detoxification of *S*-adenosylmethionine (SAM) (Schauder *et al.*, 2001). In the presence of AI-2, transcription of LEE1 operon *ler* (LEE-encoded regulator) was activated (Sperandio *et al.*, 1999), whose product Ler activates expression of other LEE genes in a regulatory cascade (Elliott *et al.*, 2000a). The *luxS*/AI-2 QS system was also shown to activate the expression of genes involved in assembly and motility of flagellae by activation of *flhDC* transcription (Sperandio *et al.*, 2001). The genes encoding the expression, assembly and motility of flagellae are also activated by QS regulators, quorum sensing *E. coli* regulator B and C (QseBC) (Sperandio, Torres, and Kaper, 2002). The QseBC activates transcription of *flh*DC regulon, however, it has no role in the expression of LEE-encoded TTSS. The QS system luxS/AI-2 also activates

transcription of *qseBC* (Sperandio, Torres, and Kaper, 2002). In EHEC and EPEC, another QS molecule, QseA, a member of LysR family, enhances expression of Ler and hence activates the transcription of other LEE genes (Sperandio, Li, and Kaper, 2002). The *qseA* mutant secreted strikingly low levels of Tir, EspA, and EspB compared to the wild type strain (86-24) (Sperandio, Li, and Kaper, 2002). In EHEC, QseA is not involved in the regulation of flagellaer regulon whereas in EPEC, it represses flagellaetion and motility (Sircili *et al.*, 2004).

However, recently Sperandio *et al.* (2003) have shown that flagellum synthesis and TTSS were regulated by another LuxS dependent novel autoinducer (AI-3) and not the AI-2. AI-3 activated the transcription of *qseBC* and LEE genes. Furthermore, epinephrine (Epi) and norepinephrine, the eukaryotic signal molecules cross-talk with bacterial QS systems regulating LEE expression and motility. The transcription of *flhDC* was activated by both Epi and/or AI-3 in a *luxS* mutant and this signalling was blocked by both α- and β- adrenergic antagonists.

1.4.3 Flagellaer-structure

The bacterial flagellum is composed of a filament that is attached to a molecular motor (the basal body and the hook complex). Flagellaer filaments are composed of 11 protofilaments, which wrap together to form the filament (Fig. 1.2). Each protofilament is composed of almost entirely flagellin monomers that have three globular domains (D1, D2 and D3), of which D1 is the most highly conserved (Fig.1.3). The protofilaments assemble through axial intermolecular contacts between the D1 domain, concave and convex surfaces on adjacent monomers

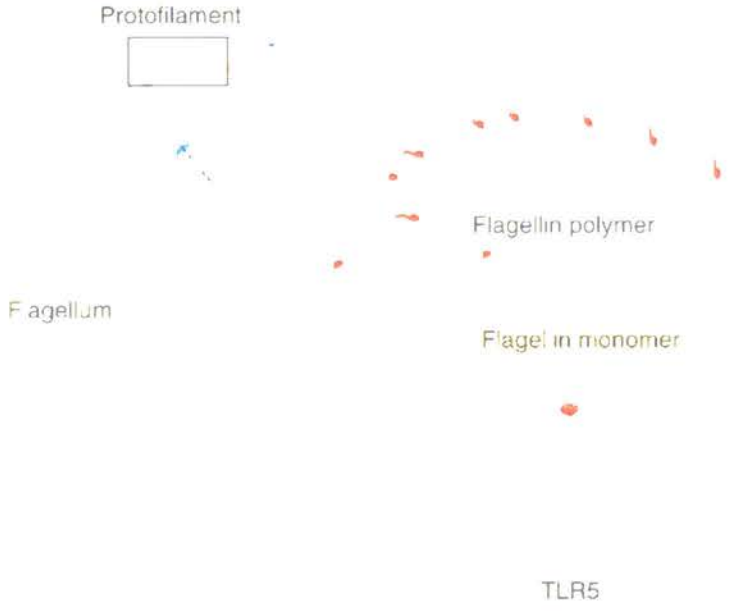

Fig. 1.2 Flagellar structure. Bacteria swim by rotating a flagellum that is attached by a flexible hook to the 'motor', part of which is a disc contained in the membrane. The propeller, up to 15 μm in length, is composed of 11 protofilaments. Each protofilament is nearly exclusively a polymer of flagellin. The monomers are packed through relatively small but deeply buried axial interactions between the concave (green) and convex (red) surfaces of the D1 domain. TLR5 recognizes the flagellin monomer at the very same surface, normally hidden in the filament, and activates the immune system (Reichhart, 2003).

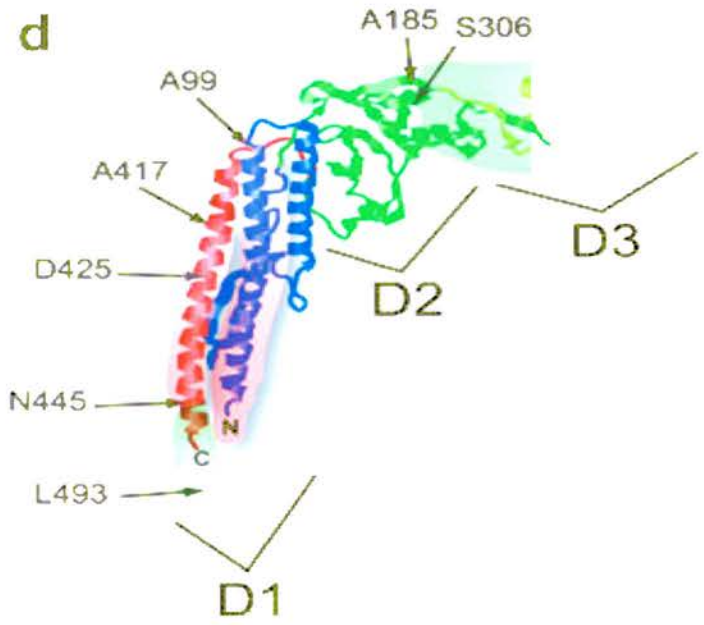

Fig. 1.3 Flagellin structure with respect to different domains. The D1 domain consists of the red and blue segments, the D2 domain is green and the D3 domain is yellow. TLR5 recognises discrete site in the D1 domain. D3 domain is exposed on the surface of the filament and have no role in TLR5 recognition (Smith *et al.*, 2003).

(Samatey *et al.*, 2001;Yonekura, Maki-Yonekura, and Namba, 2003). These D1-domain polypeptide regions are highly conserved and are essential for motility (Smith *et al.*, 2003a).

The immunogenic motifs of flagellin reside in the variable D3 domain (McDermott *et al.*, 2000;Steiner *et al.*, 2000) and the conserved D1 and D2 domains are important for innate immune recognition (Eaves-Pyles *et al.*, 2001b;Donnelly and Steiner, 2002). The D3 domain exposed at the surface of the flagellaer filament is not required for the motility and is a common target for antibody responses (Smith *et al.*, 2003b). Its high degree of variability indicates that D3 domain has evolved to permit considerable structural heterogeneity to evade adaptive immune responses and potentially to target specific ligands on the cell surfaces.

1.4.4 Flagellaer-epithelial interactions

To discriminate non-self from self, the innate immune system uses a set of germline-encoded pattern-recognition receptors (PRRs) that recognize conserved microbial structures called pathogen-associated molecular patterns (PAMPs) (Janeway, Jr., 2001). In humans and mice, the Toll-like receptors (TLRs), form a family of ten members that typically represent such PRRs. These proteins consist of an extra-cellular domain with a leucine-rich repeat, one or two cysteine-rich regions, and a cytoplasmic region called the Toll/IL-1 receptor domain.

Flagellae from various bacterial species share conserved immunostimulatory domains that interact with TLR5 (Donnelly and Steiner, 2002;Murthy *et al.*, 2004), binding of which transduces signal cascades, involving adaptor protein MyD88 (myeloid differentiation factor 88) and IL-1 receptor-associated kinase (Hayashi *et al.*, 2001a;Moors, Li, and Mizel, 2001;Mizel and Snipes, 2002). This leads to activation of NF-κB- mediated gene expression of a diverse array of innate immune defences, including antimicrobial peptides (Ogushi *et al.*, 2004), mucin (McNamara *et al.*, 2001a), nitric oxide (Eaves-Pyles *et al.*, 2001a) and other pro-inflammatory effectors such as IL-8 (Zhou *et al.*, 2003a) and MIP3α (Izadpanah *et al.*, 2001). A recent study has shown that NF-κB-independent MEK (mitogen activated protein-

kinase)-mediated signalling pathways are also involved in flagellin/TLR-5 induced IL-8 and MIP3α expression (Rhee *et al.*, 2004).

TLR5 is expressed on the basolateral surface of intestinal epithelial cells where it interacts with flagellin that has been internalized, but not with the filamentous flagellae at the apical surface (Gewirtz *et al.*, 2001a). Flagellin interacts with TLR5 via conserved sequences in the D1 domain. The deletion of these N-terminal sequences in *Salmonella typhimurium* FliC flagellin monomers prevented the TLR5 recognition (Smith *et al.*, 2003a). The TLR5 binding motifs of flagellaer-D1 domain are cryptic and are exposed on the surface of flagellin monomers only. In one study filamentous flagellin TLR5-stimulatory activity was reduced by 96% compared with that of monomeric flagellin (Smith *et al.*, 2003a).

Many additional non-TLR5 receptors have also been proposed as complementary means of flagellae-epithelium interaction. Recently Rhee et al. (Rhee *et al.*, 2004) have shown that *Pseudomonas aeruginosa* flagellae bound initially to asialo-GM1 on the apical surface of the epithelial cells. It was only after 4 h of exposure to flagellae that TLR5 expression was induced and mobilized to apical surface where it colocalized with the adherent flagellae. Other epithelial (co-)receptors for flagellae include TLR2 (Adamo *et al.*, 2004), gangliosides (McNamara *et al.*, 2001b) (Ogushi *et al.*, 2004) and mucin (Lillehoj, Kim, and Kim, 2002). In addition to their involvement in inducing inflammation, a role in adherence of bacteria to epithelial cells has been described for certain flagellae (including *Clostridium difficile* (Tasteyre *et al.*, 2001), *Burkholderia pseudomallei* (Inglis *et al.*, 2003), *Aeromonas* spp. (Kirov, Castrisios, and Shaw, 2004b) and *Listeria monocytogenes* (Dons *et al.*, 2004). Flagellae have also been implicated in pathogenicity of enteropathogenic *E. coli* (EPEC; a pathotype related to EHEC) (Giron *et al.*, 2002) for which roles in initial adherence, actin rearrangement and microcolony formation on HeLa cells were demonstrated. Hence a role was proposed for flagellae in adherence and anchoring of EPEC to epithelium.

1.5 Bacterial adherence to cellular receptors

The ability to adhere to host surfaces is a necessary step in the successful colonization and ultimately production of disease by microbial pathogens. Protein-carbohydrate interactions play a critical role in adherence of pathogens to epithelial surfaces (Boren *et al.*, 1993;Finlay, Heffron, and Falkow, 1989;Roberts *et al.*, 1994;Saiman and Prince, 1993;Sharon, 1987). Colonization or invasion of gastrointestinal mucosal surfaces by enteric pathogens often involves microbial lectin-like adhesins that recognize specific glycoconjugates on intestinal epithelial cells (Hultgren *et al.*, 1993;Sharon, 1987). In the mammalian intestine the carbohydrate structures on epithelial cell surfaces vary amongst species, intestinal regions, cell types and even among enterocytes on a single villus. The selectivity of microbial adhesins for specific carbohydrate moieties (Table 1.4) may determine the patterns of susceptibility of certain species and individuals and the regional distribution of pathogens in the host gastrointestinal tract (Boren *et al.*, 1993;Hultgren *et al.*, 1993;Lindstedt *et al.*, 1991). The diversity of oligosaccharide chains present on mammalian cells can result from cell-type or animal species-specific regulation of glycotransferases, involved in the biosynthesis of glycoprotein and glycolipids (Varki, 1996). Multicellular organisms are constantly changing surface expression of these contact points as an evolutionary process to evade the infections. e.g. NeuGc, a species of sialic acid is present in most of mammals including apes except for humans which express NeuAc, the non-hydroxylated form, due to an inactive hydroxylase. *E. coli* K-99 an important enteric pathogen in calves and pigs, requires NeuGc for intestinal adhesion (Mouricout *et al.*, 1990) adhesion is thus non-pathogenic to humans. Such heterogenieity in glycoconjugate expression could influence EHEC binding and infectivity for different hosts.

Table 1.4: Selected bacterial fimbrial adhesins with their respective glycoconjugate(s) receptors (adapted from (Klemm and Schembri, 2000)).

Organism	Fimbriae	Adhesin	Receptor or affinity for	Reference
E. coli	Type 1	FimH	Mannosides, laminin, fibronectin, plasminogen	(Krogfelt, Bergmans, and Klemm, 1990;Pouttu *et al.*, 1999;Sokurenko *et al.*, 1998)
E. coli	P fimbriae	PapG	Galα(1-4)Gal moity in globoseries of glycolipids	(Hull *et al.*, 1981)
E. coli	Prs fimbriae	PrsG	Galα(1-4)Gal moity in globoseries of glycolipids	(Lund *et al.*, 1988)
E. coli	S fimbriae	SfaS	α-sialyl-(2-3)-β-galactose	(Korhonen *et al.*, 1984)
E. coli (UPEC)	F 1 C fimbriae	FocH	N-acetylgalactosamine, galactose,glycophorin	(Riegman *et al.*, 1990)
E. coli (ETEC)	K88	FaeG	Galα(1-3)Gal, Galβ, GlcNAc, fucose, polymycin B, nonapeptide	(Smit *et al.*, 1984)
E. coli (ETEC)	K99	FanC	NeuGe-GM3, NeuGc-SPG, sialoglycoproteins	(Erickson *et al.*, 1994)
E. coli (ETEC)	CFA/1	CfaB	NeuAc-GM2, human erythrocyte sialylglycoprotein	(Jordi *et al.*, 1992)
Proteus mirabilis	MR/P fimbriae	MrpH	Galα(1-4)Gal moity in globoseries of glycolipids	(Li, Johnson, and Mobley, 1999)

1.6 Follicular associated epithelium (FAE): a potential site for adherence of enteropathogens

The epithelium overlying the follicles (FAE) is characterised by presence of structures that are distinct from the surrounding villi. Unlike villus epithelium, FAE contains very few or no goblet or enteroendocrine cells, fewer defensin- and lysozyme-producing Paneth cells and lower membrane-associated hydrolases. FAE is devoid of polymeric immunoglobulin receptor and therefore not able to transport protective IgA from the interstitium to the lumen. The glycocalyx of FAE presents an array of glycoconjugates different from the surrounding villus epithelium (Giannasca *et al.*, 1999;Giannasca *et al.*, 1994;Gebert and Hach, 1993b;Pappo, Steger, and Owen, 1988). All these features tend to promote local contact of pathogens and intact antigens with FAE, one of its functions.

Table 1.5: Interaction of various enteric bacterial pathogens with M-cells (adapted from (Finlay and Falkow, 1989).

Bacterium	Adhesin	Host Receptor	Type of interaction to M-cells	Reference
E. coli RDEC-1	AF/R1	NK	Microvillus effacement, pedestal formation, no invasion	(Inman and Cantey, 1983;Von Moll and Cantey, 1997)
Vibrio cholerae	NK	NK	Phagocyte-like uptake into vacuole	(Owen *et al.*, 1986)
Yersinia enterocolitica and *Y. pseudotuberculosis*	Invasin	β-1 integrin	Active invasion into vacuole	(Clark, Hirst, and Jepson, 1998)
Shigella flexneri	IpaB-D	α5β1 integrin	Invasion into vacuole, then release in to cytoplasm and intercellular spread, M-cell destruction	(Watarai, Funato, and Sasakawa, 1996)
Campylobacter jejuni	NK	NK	Active invasion in to vacuole	(Walker *et al.*, 1988)
Salmonella typhi and *S. typhimurium*	Lpf	NK	Ruffling, microvillus degeneration, uptake into vacuole, followed by M-cell destruction	(Baumler, Tsolis, and Heffron, 1996)

NK- not known

The FAE contains specialized epithelial M-cells that sample antigens from the lumen directly to the intraepithelial lymphoid cells and to subepithelial organised lymphoid tissue, initiating a protective immune response (Giannasca *et al.*, 1994). M-cells generally lack an organised brush border and a well-defined filamentous brush border glycocalyx (FBBG). Their apical surfaces have only a thin (20-30 nm) glycoprotein coat (Frey *et al.*, 1996a) in comparison to villus enterocytes which have a highly differentiated structure consisting of rigid, closely packed microvilli coated with a 400-500 nm thick FBBG composed of highly glycosylated transmembrane mucins. Therefore, though the M-cells are crucial for induction of protective mucosal immune responses, these also provide portals of entry to many enteropathogens.

The availability and accessibility of different glycoconjugates, potential ligands for the bacterial lectin-like adhesins, facilitates adherence of many enteric pathogens to FAE. The diversity of glycoconjugates and apical membrane proteins including β-1

integrins may allow M-cells in the FAE to sample a wide variety of microbes in the gut to induce protective immune responses. However, certain pathogenic organisms have evolved diverse strategies to invade via mucosal surfaces, including selective adherence to FAE and M-cells (Table 1.5). It is likely that adherence and uptake of microorganisms by M-cells involves a sequence of molecular interactions including initial recognition of M-cell surface oligosaccharides by bacterial lectin-like adhesins, followed by more intimate association that would require expression of other additional virulence determinants, processing of M-cell surface molecules and recruitment of integral membrane proteins of M-cells to the site of attachment.

1.6.1 EHEC adherence to FAE

E. coli O157:H7 showed a distinct tropism and formed A/E lesion on the FAE of ileal Peyer's patches in *in vitro* organ culture assays using human intestinal explants (Phillips *et al.*, 2000b). The FAE-restricted tissue tropism of *E. coli* O157:H7 was associated with the type of intimin expressed. Following expression of intimin γ from EHEC in the EPEC background, a restricted pattern of tissue tropism towards Peyer's patches was observed when tested on human intestinal explants (Fitzhenry *et al.*, 2002b). From the recently published complete gene sequence of O157:H7, several putative fimbrial operons (Perna *et al.*, 2001) including long polar fimbriae (Lpf) (Torres *et al.*, 2002a) have been identified. The Lpf in *Salmonella enterica* serovar Typhimurium restricts its binding to Peyer's patches (Baumler, Tsolis, and Heffron, 1996) and hence Lpf along with the intimin may drive the FAE specific tropism of *E. coli* O157:H7.

EHEC (*E. coli* O157:H7) colonization site in cattle

Natural and experimental infection of normal cattle with *E. coli* O157:H7 results in efficient colonization of the intestinal tract in the absence of clinical signs. In contrast, EHEC serogroups O5, O26, and O118 are commonly associated with diarrhea in farm animals, which imposes a significant economic burden on livestock producers (Pearson et al., 1999; Weiler et al. 1996; Stevens et al., 2002). Extensive adherence and AE-lesion formation occur with O5, O26, and O111 in the bovine large intestine (Pearson et al., 1999; Stevens et al., 2002), whereas EHEC O157 has

been reported to exhibit tropism for lymphoid follicle-dense mucosa in the terminal rectum adjacent to the recto-anal junction (Naylor et al., 2003). The terminal region of the cattle rectum is characterised by the presence of a high density of lymphoid follicles. Since many of the enteric pathogens have been documented to interact with the FAE/M-cells it is reasonable to postulate that epithelium associated with lymphoid dense tissue in the terminal rectum may be crucial in interaction of *E. coli* O157:H7 with cattle. The local environment at the site may be conducive for the expression of certain bacterial adhesins, and the availability and accessibility of distinct glycoconjugates and other integral membrane proteins on FAE or M-cells may be the determining factors for *E. coli* O157 colonization of the terminal rectum of cattle.

Together, these observations suggest that EHEC O157:H7 and non-O157:H7 may colonize the bovine intestines by distinct mechanisms. Strategies to lower the prevalence of EHEC in cattle offer the possibility of reducing the incidence of human infections. An understanding of the mechanisms by which EHEC colonizes the ruminant intestines is necessary for the development of effective intervention strategies.

It is clear that EHEC interaction with intestinal epithelium is a complex phenomenon and involves multiple bacterial and host determinants. The factors playing a role in *E. coli* O157:H7 colonization in cattle by and large are obscure therefore the aim of this research was to gain further understanding of the molecular basis of terminal rectum-specific tropism of *E. coli* O157:H7 in cattle with a particular focus on host determinants.

Aims

1. To characterise epithelium and associated lymphoid follicles for features that may determine the bovine terminal rectal tissue-specific tropism of *E. coli* O157:H7 in cattle, using immuno-histochemistry, lectin binding and ultra-microscopy techniques.

2. To develop and characterise primary epithelial cell culture from the bovine terminal and mid-rectal regions (0-3 cm and 18-20 cm proximal to recto-anal junction, respectively) as an *in vitro* model to examine EHEC interaction with bovine intestinal epithelium.
3. To determine the role(s) of verotoxin in interaction of EHEC strains with bovine rectal epithelium using primary epithelial cell culture as an *in vitro* model since verotoxin is a major secreted virulence factor of EHEC that could significantly affect colonisation.
4. To assess the role(s) of flagellae in adherence of *E. coli* O157:H7 to bovine rectal epithelium as the surface organelles are important determinants of interaction with epithelium and induction of host inflammatory responses.

2 Phenotypic and functional characterisation of follicle-associated epithelium of rectal lymphoid tissue

2.1 Introduction

The mammalian intestinal surface is exposed to a multiplicity of components in the luminal contents. Among important functions of the epithelium is prevention of infection by potentially pathogenic microorganisms. Factors such as brush border glycocalyx (Frey *et al.*, 1996b), are involved in limiting access of microbes, their components, or other potentially detrimental factors to the epithelial surface whilst intraepithelial tight junctions (Madara *et al.*, 1990) prevent their access to sub-epithelial tissues. Other factors secreted by the epithelium are also important in protection of mucosal surfaces including mucins, antimicrobial peptides and antimicrobial enzymes, for instance (Ouellette and Selsted, 1996;Mashimo *et al.*, 1996). To complement innate defences, antigen-specific defences including immunoglobins secreted via epithelium are also activated hence the adaptive immune system must sample antigens in order to mount anti-microbial, protective responses. Mechanisms have evolved whereby particulate material and macromolecules can be transported across intact mucosal surfaces for presentation to lymphoid tissue. This function is performed principally by M-cells, membranous or microfold cells (Owen and Jones, 1974) but also by other cells such as dendritic cells (Rescigno *et al.*, 2001) of the reticulo-endothelial system that are typically situated in the epithelium overlying organised foci of lymphocytes known as lymphoid follicles (LF).

Organisation and composition of intestinal lymphoid follicles (collectively termed gut-associated lymphoid tissue – GALT) varies between different regions of the intestine within species and also between species. Gut LF may be distributed as discrete, individual follicles or form aggregates at anatomically defined sites, for example the Peyer's patches of the terminal ileum (Nagi and Babiuk, 1989;Nagi and Babiuk, 1988). The characteristics of epithelial cells overlying LFs are distinct from other epithelia. These contain M-cells as a significant component. Reflecting the heterogeneity in GALT, the follicle-associated epithelium (FAE) overlying LFs exhibits heterogeneity in characteristics including general morphology, the

proportion of M-cells, their ultrastructure and the complement of surface receptors (Jepson *et al.*, 1992;Gebert, Hach, and Bartels, 1992). For example, in comparison to gut enterocytes, FAE, and in particular M-cells, express reduced glycocalyx (Frey *et al.*, 1996a) and readily take up foreign particles and micro-organisms.

Several micro-organisms exploit FAE or M-cells for attachment or invasion. For instance, certain viruses including HIV-1 (Amerongen *et al.*, 1991;Owen, 1998), reovirus type-1 and poliovirus (Wolf *et al.*, 1981), enteroinvasive bacteria including *Shigella flexneri* (Perdomo *et al.*, 1994), *Salmonella enterica* serovars *Typhi* and *Typhimurium* (Jones, Ghori, and Falkow, 1994;Kohbata, Yokoyama, and Yabuuchi, 1986), *Yersinia enterocolitica* and *Y. pseudotuberculosis* (Gebert *et al.*, 1999;Grutzkau *et al.*, 1990) and extracellular bacterial pathogens including *Vibrio cholerae* (Owen *et al.*, 1986), *Campylobacter jejuni* (Walker *et al.*, 1988) and *Escherichia coli* RDEC-1 (Inman and Cantey, 1983) target FAE/M-cells during infection. Recent reports that verotoxigenic *E. coli* O157:H7 localises to FAE of the terminal rectum of cattle, its main reservoir host, within a small region defined by the presence of lymphoid aggregates (Naylor *et al.*, 2003;Rice *et al.*, 2003a;Sheng *et al.*, 2004a) is consistent with the tropism for FAE displayed by other enteric bacteria. Therefore FAE, and in particular M-cells, are important interfaces between the intestine and micro-organisms.

As a result of the importance of M-cells and FAE in interaction between host and pathogens, the objective of this work was the characterisation of the lymphoid tissue and associated epithelium of the bovine terminal rectum. Specifically, investigation included characterisation of general morphology, ultrastructure, lymphoreticular cell populations within the lymphoid follicles and M-cell function in order to further the understanding of the biology and role of this site.

2.2 Materials and Methods

2.2.1 Tissue sampling and gross evaluation of lymphoid follicle distribution

Tissues for all procedures were obtained either from the local abattoir or from animals being used for other experimental purposes and were transported to the laboratory in ice cold Hank's Balanced Salt Solution (HBSS, GIBCO BRL). For tissue obtained from the abattoir, typically 30-45 minutes elapsed between tissue excision and initiation of experimental protocols whilst for tissue obtained from experimental animals this period was typically 15 minutes. Previous investigations have shown that gastrointestinal tissue integrity and function is retained for at least 3 h after removal (Jackson *et al.*, 2004). Procedures with animals were subject to ethical review and were performed with appropriate licencing from the Home Office and in accordance with the Animal (Scientific Procedures) Act of the UK.

To assess the distribution of lymphoid follicles macroscopically in the bovine terminal rectum, tissue was treated with acetic acid (adapted from (Chauhan, 1970;Cornes, 1965)). The terminal 30 cm of rectum and anal canal were obtained from 5 mature cattle at a local abattoir. In the laboratory, the specimens were opened longitudinally and the entire mucosal surface was dissected from the sub-mucosa and immersed in 70% acetic acid (v/v) overnight. Lymphoid follicles became visible as white nodules on the sub-mucosal surface. The distribution of lymphoid follicles was the same in all animals examined and representative results are presented.

2.2.2 Processing for Histology

The terminal 30 cm of rectum was obtained at necropsy from four male, clinically healthy, Holstein-Friesian calves aged between 8 and 12 weeks. For immunohistochemistry, full-thickness tissue from the regions 0-2 cm and 18-20 cm proximal to the recto-anal junction (RAJ) were fixed in zinc acetate fixative (ZSF) (Gonzalez *et al.*, 2001) (0.1M Tris-base buffer, calcium acetate (0.05% w/v), zinc acetate (0.5% w/v) and zinc chloride (0.5% w/v); pH 7.4). After 6-8 h of fixation, the tissue samples were trimmed to 3-4 mm thickness and further fixed overnight in ZSF at room temperature (RT). The fixed tissue samples were transferred to 70% ethanol (v/v) before processing to paraffin wax. Sections (5 μm) were cut and mounted on

coated slides (Superfrost Plus, Menzel Glaser, Germany) and dried overnight at 37°C. For cryosectioning, tissue samples were immersed in Tissue-TEK optimum cutting temperature embedding medium (OCT; BDH; Poole,UK), snap frozen in liquid nitrogen then stored at –20°C. Frozen sections were cut at 8-10 μm (Shandon Cryostat, UK) and mounted on Superfrost microscope slides. The cryosections were air-dried, fixed in methanol cooled to -20°C then rehydrated for 10 minutes in PBS prior to staining.

2.2.3 Immunohistochemistry

Sections from paraffin-embedded tissue were de-waxed and re-hydrated in graded alcohols. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 minutes. After treatment with 25% v/v normal goat serum (Vector Laboratories Inc., Burlingame USA) for 30 minutes, sections were incubated (overnight at 4°C) using a panel of monoclonal antibodies specific to various cell markers (Table 2.1). Primary antibody was detected using goat anti-mouse peroxidase-labelled antibody (Horizontal En Vision Plus HRP system, Dako, Ely, UK) (30 minutes incubation at room temperature). The sections were developed in a citrate buffer containing 3,3' diaminobenzidine hydrochloride (DAB - Vector Laboratories Inc., Burlingame USA) for 7-8 minutes, counterstained with haematoxylin and mounted in DPX (BDH Laboratory Supplies Poole, UK). Tris-buffered saline was used to wash tissue sections between each stage of the labelling procedure and as a diluent for the antibodies.

For lectin binding studies, a panel of seven biotinylated lectins (Vector Laboratories, Inc Burlingame USA) (Table 2.2) were used on frozen sections, ZSF-fixed or paraformaldehyde-fixed sections. After blocking of endogenous peroxidase activity and non-specific binding (as described above) sections were incubated with biotinylated lectins (1 h at room temperature). The sections were developed with streptavidin-HRP conjugate and DAB and counterstained with haematoxylin as above. Sections were washed in PBS (3 x 5 minutes) between each step.

Histological characteristics, lectin and immunohistochemical staining of rectal tissues were consistent between animals therefore representative results are provided.

Table 2.1: Monoclonal antibodies used in immunohistological staining

Monoclonal Antibody	Specificity	Cellular Expression	Source	Reference
CC21	CD21	Follicular Dendritic Cells, Mature B Cells	IAH, Compton	(Naessens and Howard, 1991)
CC20	Bovine CD1b	Dendritic Cells	IAH, Compton	(Howard *et al.*, 1993)
CC15	Bovine WC1	γδ T Cell	IAH, Compton	(Howard *et al.*, 1989)
ILA-12	CD4	T helper Cells	ILRAD, Nairobi, Kenya	(Teale *et al.*, 1987)
ILA-51	CD8	Cytotoxic T Cells	ILRAD, Nairobi, Kenya	(Teale *et al.*, 1987)
ILA-156	CD40	B cells, Antigen Presenting Cells	ILRAD, Nairobi, Kenya	(Norimatsu *et al.*, 2003)
ILA-111	CD25	Activated T and B Cells, Macrophages	ILRAD, Nairobi, Kenya	
ILA-30	Ig M	Ig M and Plasma cell	ILRAD, Nairobi, Kenya	(Levkut *et al.*, 1995)
K84 2F9	Ig A	IgA and Plasma cells	Serotec Ltd, Oxford	
ILA-43	CD2	αβT cells, Natural Killer cells	ILRAD, Nairobi, Kenya	(Teale *et al.*, 1987)
ILA-24	MHC-II	Dendritic cells, B cells, Macrophages and other Antigen presenting cells	ILRAD, Nairobi, Kenya	
Anti vimentin	Vimentin	Cells of mesenchymal origin, M cells	Sigma-Aldrich	(Gebert, Hach, and Bartels, 1992)

IAH - Institute for Animal Health, Compton, UK
ILRAD - International Laboratory for Research on Animal Diseases (now ILRI), Nairobi, Kenya

Table 2.2: Lectins used in this study

Lectin	Carbohydrate Specificity	Species/Site Specificity
UEA-1 (*Ulex europaeus*)	α-L-fucose	M cells in cecum (Gebert and Hach, 1993a) and palatine tonsils (Gebert, 1997) of rabbit; Mouse Peyer's patch M cells (Clark *et al.*, 1993).
WGA (*Triticum vulgaris*)	D-N-acetyl glucosamine	M cells in cecum of rabbit (Gebert and Hach, 1993a) and chicken (Jeurissen, Wagenaar, and Janse, 1999); NALT of rat (Takata, Ohtani, and Watanabe, 2000).
DBA (*Dolichos biflorus*)	α-D-galNAc	Rabbit caecal M cells (Gebert and Hach, 1993a).
(PNA) (*Arachis hypogaea*)	D-galactose	Not Determined
RCA (*Ricinus communis*)	β-D-galactose	Not Determined
SBA (*Glycine max*)	D-N-acetyl galactosamine	M cells in cecum of rabbit (Gebert and Hach, 1993a) and chicken (Jeurissen, Wagenaar, and Janse, 1999).
Con A (*Canavalia ensiformis*)	α-D-mannose	Not Determined

2.2.4 Electron Microscopy

Samples of tissue from 0-2 cm and 18-20 cm proximal to the RAJ were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). For transmission electron microscopy (TEM) the specimens were post-fixed in a mixture of 1% osmium tetroxide (w/v) and 1.5% potassium ferricyanide (w/v), dehydrated in a graded series of alcohols and embedded in Epon 812 (Fluka). Thick sections (1 μm) were stained with 1% toluidine blue to identify regions containing follicle-associated epithelium from which 70-90 nm ultrathin sections were cut. The sections were stained with uranyl acetate and lead citrate and examined on a Philips CM12 transmission electron microscope. For scanning electron microscopy (SEM), fixed tissue was dehydrated in graded acetones, critical point dried, sputter coated with gold/palladium and viewed on a Hitachi 4700 FEG SEM (Field Emission Gun Scanning Electron Microscope) scanning electron microscope. Characteristics were consistent in tissues obtained from separate animals and representative results are provided.

2.2.5 Microparticle Uptake *in vitro* and *in vivo*

Tissue specimens obtained from adult cattle at a local abattoir were transported in ice cold HBSS. The terminal rectal mucosa 3 cm proximal to the RAJ was carefully

excised and washed in cold PBS, cut into 1 cm squares with a thickness of 2 mm and placed in RPMI 1640 tissue culture medium (Sigma-Aldrich). The mucosal pieces were placed on a sterile foam pad immersed in prewarmed (37°C) RPMI 1640. An agarose (3% w/v in PBS) collar was placed around the explant using molten agarose to seal any gaps between the collar and edges of the tissue.

Aliquots (100 µl) of 1:100 suspensions of latex microspheres of sizes 0.2 µm or 0.5 µm (Polysciences Inc., Germany) were pipetted evenly onto the mucosal explants. These explants were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 45 minutes. The explants were washed thoroughly 3 times in PBS then were placed in OCT and processed for cryo-sectioning as described above. The cryosections were air dried overnight; fixed and permeabilized in 2% PFA/Triton X-100 (0.25% v/v) for 15 minutes at room temperature. After washing with PBS the sections were stained with phalloidin-TRITC (5 µg/ml w/v, Sigma) for 20 minutes in the dark. Sections from two animals were examined by confocal microscopy and representative images are presented.

To assess bead uptake *in vivo*, 500 µl suspension of 0.5 µm latex microspheres diluted as above in PBS were delivered intra-rectally to clinically healthy, Holstein-Friesian calves aged between 8 and 12 weeks. Tissue was collected from these calves at 20 minutes intervals from 30 minutes after following euthanasia with intravenous pentobarbital. The abdomen was opened and the terminal 10 cm of rectum and anus were removed as a single piece after the rectum was double ligated and transected, the anus circumsected, and the pubic bone reflected. Tissue sections from 0-2 cm proximal to RAJ were fixed in ZSF processed for cryo-sectioning as described above. All procedures with animals were subject to ethical review and were performed with appropriate licencing from the Home Office and in accordance with the Animal (Scientific Procedures) Act of the UK.

2.2.6 Confocal Microscopy

The Leica TCS NT confocal system was used to acquire a series of optical sections. Where required, images were visualised in the Surpass module of the Imaris software

suite (Bitplane AG, Zurich), which converts the image voxels into a geometric object. Following threshold segmentation, the image was then surface rendered allowing volumes and interactions between objects to be defined.

2.3 Results

2.3.1 Distribution and morphology of lymphoid follicles in the bovine terminal rectum

The majority of the rectal mucosa formed longitudinal and transverse folds and ridges. The mucosa became smoother within a region 4-5 cm cranial to the RAJ (defined by the junction between columnar and stratified squamous epithelium) and longitudinal folds termed the ampulla recti were present. In this region high densities of lymphoid follicles formed confluent patches with solitary follicles becoming more scarce cranially (Fig. 2.1A). Lymphoid follicles were rare beyond 5 cm cranial to the RAJ. There was no association between the lymphoid patches and the longitudinal folds at this site.

The lymphoid follicles at the terminal rectum were observed in two morphologically distinct forms: those showing a single germinal centre capped by a dome layer that did not extend above the level of the surrounding mucosa (Fig. 2.1B) and those showing several contiguous follicles underlying a pit or diverticulum formed by an inversion of the mucosal surface (Fig. 2.1C). Protruding dome-like structures lined by FAE were frequently present within these pits (Fig. 2.1D).

2.3.2 Lymphoreticular cell populations within the lymphoid follicles

Immunohistochemistry was employed to identify and map the distribution of several classes of lymphocytes and antigen-presenting cells classically associated with sites of antigen sampling and presentation. Represenative results are presented in Fig. 2.2. $CD4^+$ T lymphocytes were distributed predominantly in the parafollicular zones although they were also observed within follicles (Fig. 2.2A). CD8+ lymphocytes were scarcely scattered throughout follicles (Fig. 2.2B). Few WC1+ $\gamma\delta$ T-cells were observed infiltrating the lamina propria away from the follicle (Fig. 2.2C) but not in the follicle itself (not shown). CD2-positive cells ($\alpha\beta$ T-cells or natural killer cells)

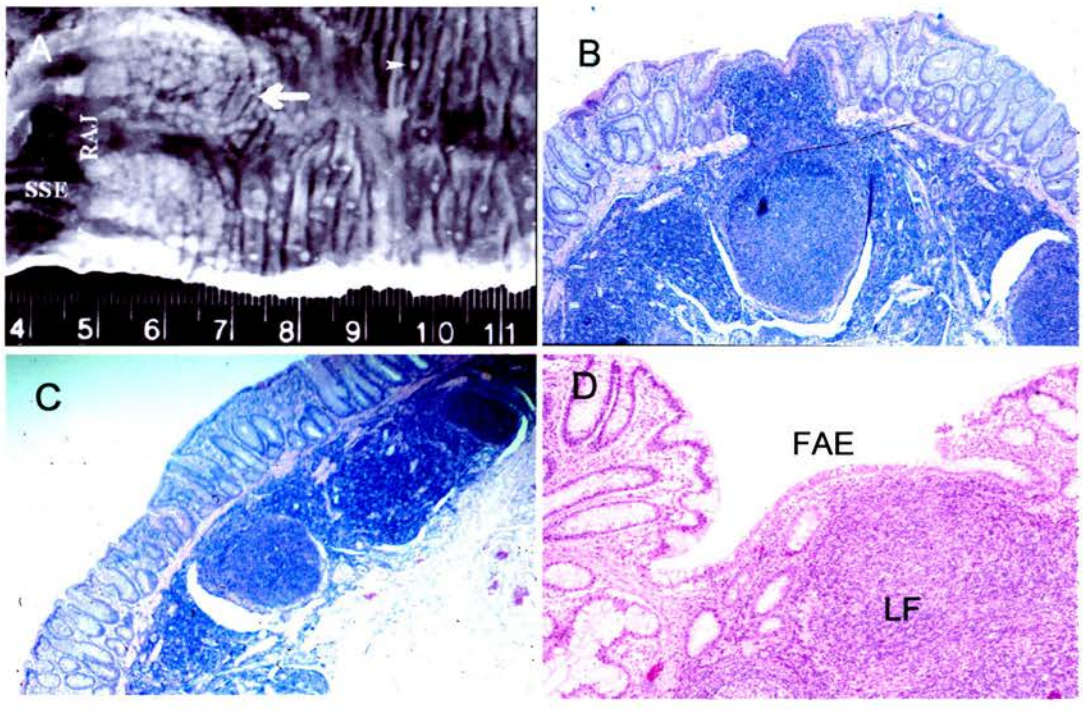

Fig. 2.1 Characterisation of the bovine terminal rectal mucosa. Fig.2.1A shows the terminal portion of rectal mucosa on treatment with 70% v/v acetic acid and debridement of serosal connective tissue. Anal canal and recto-anal junction (RAJ) distal to ampulla recti can be seen clearly. The aggregates of lymphoid follicles (arrows) are observed in high density in the region 0-3 cm proximal to recto-anal junction and individual follicles (arrow head) are visible as discrete white nodules. SSE - stratified squamous epithelium. This distribution of lymphoid follicles is representative of anal-rectal mucosa of all animals examined. Fig. 2.1B-D are hematoxylin and eosin (H&E) stained sections of bovine rectal lymphoid follicles (LF). (B) a lymphoid follicle in the submucosa extending through the muscularis mucosae into the lamina propria (25x magnification). (C) contiguous lymphoid follicles completely located under the muscularis mucosae (25x magnification). These have been previously referred to as propria nodule and lymphoglandular complex respectively. (D) The follicles form a blunt elevation into the gut lumen, covered by a layer of epithelial cells, the follicular associated epithelium (FAE) (100x magnification). Goblet cells are scarce in the FAE

were common within the parafollicular region and a few were also present within follicles (Fig. 2.2D). Cells expressing CD21 (mature B-cells and follicular dendritic cells) were present in the light zone of the germinal centre of the lymphoid follicles (Fig. 2.2E). CD40-expressing cells (B-cells or antigen presenting cells) were present in the germinal centres of follicles (Fig. 2.2F). Some of these cells, presumably including antigen-presenting cells, were also observed in close proximity to the FAE. IgA- and IgM-expressing cells were present throughout follicles and the lamina propria indicating the presence of activated B-cells and plasma cells (not shown). MHC class II-expressing cells (not shown) were present on a large population of cells both within the follicles and in the parafollicular region. CD1b-dendritic cells were seen either scattered throughout the lamina propria or in small clusters in the proximity of lymphoid follicles (not shown).

2.3.3 Characteristics of Follicle Associated Epithelium

The FAE was characterised by immuno-histochemical staining for vimentin, an intermediate filament of the cytoskeleton. The majority of cells within the FAE and follicle-associated crypts from the bovine terminal rectum stained strongly for vimentin (Fig. 2.3A) – in these regions, typically >60% of epithelial cells were positive. Vimentin expression is a characteristic of M-cells, but not other epithelial cell types thus suggesting that a substantial proportion of FAE was composed of M-cells. Epithelium not associated with LFs (in this instance rectal mucosa obtained from approx. 20cm proximal to anal-rectal junction) did not express vimentin, although occasional areas stained weakly (Fig. 2.3B). In all immunoreactive cells, vimentin was localised predominantly in the perinuclear cytoplasm of the cells. Many of the vimentin-positive cells appeared to contain basolateral indentation(s) enclosing lymphocytes, another characteristic of M-cells.

Scanning and transmission electron microscopy (TEM and SEM) of a follicle-rich area of the terminal rectum exhibited heterogeneous surface morphology with a mixed cell population (Fig. 2.4). In the region immediately overlying lymphoid follicles the majority of FAE cells were characterised by short, sparse and irregular microvilli indicative of M-cells or fused/flattened microfolds and prominent

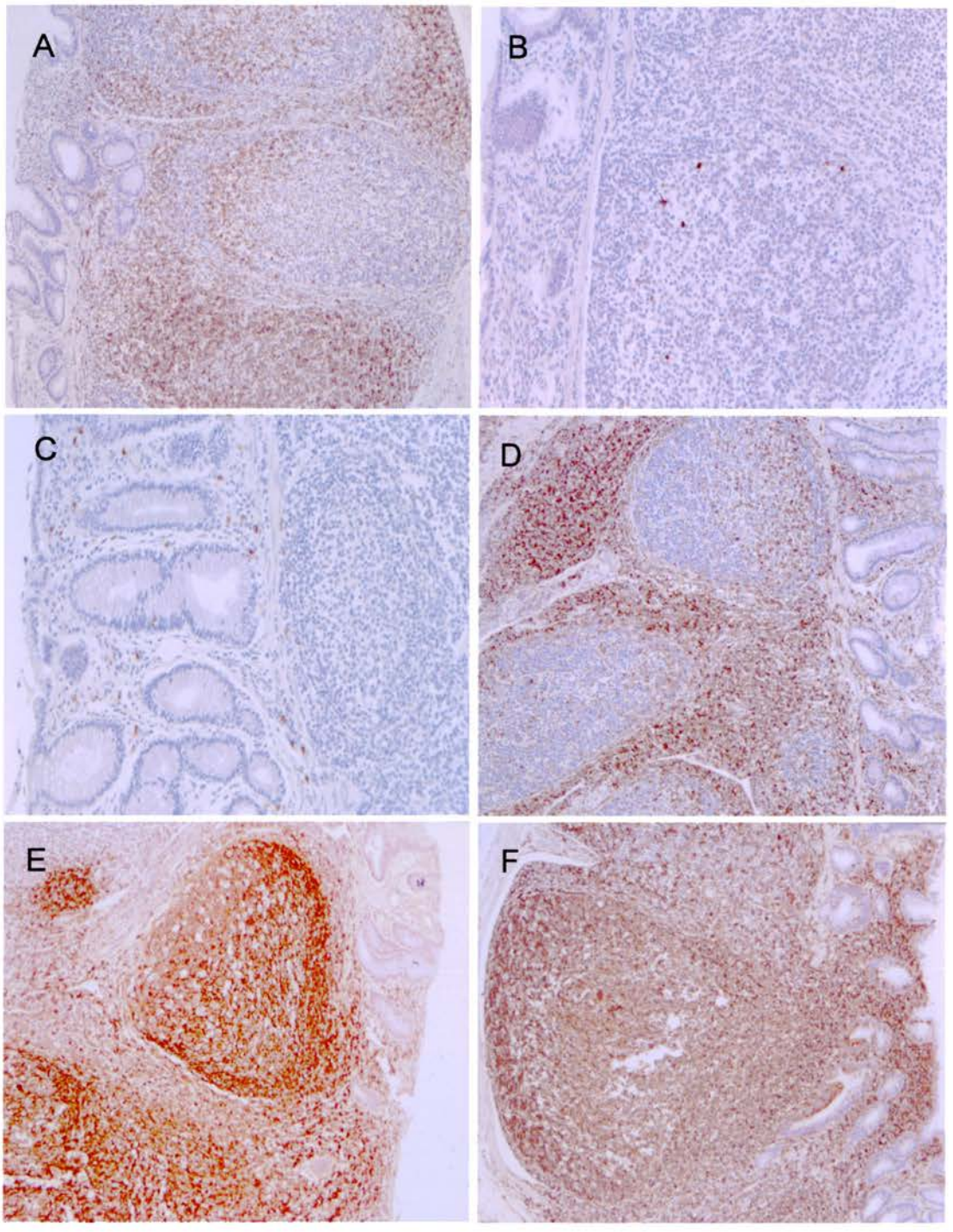

Fig. 2.2 Immunohistological analysis of cell surface antigens on lymphocytes associated with the lymphoid follicles of the bovine terminal rectal mucosa. The images illustrate the distribution of (A) T-helper cells (CD4), (B) cyototoxic T-cells (CD8), (C) WC1+ γδ T-cells, (D) αβ-T cells and natural killer cells (CD2), (E) mature B-cells and follicular dendritic cells (CD21) and (F) B-cells and antigen presenting cells (CD40). B-lymphocytes were found mainly within the follicles and T-lymphocytes were distributed in the parafollicular region. (250x magnification).

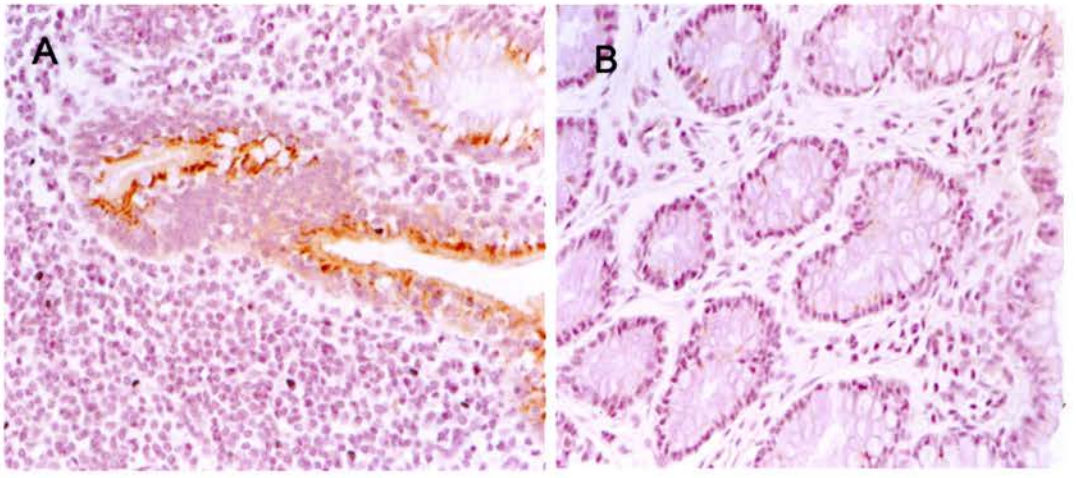

Fig. 2.3 Immunoperoxidase staining of section of bovine terminal rectal mucosa. The FAE and cells in the crypt in proximity to the follicle stain strongly for vimentin (A) whereas only few cells in crypts distant from the follicle (B) show weak staining for the vimentin. (400x magnification). Results are representative of tissues from all animals examined in the study.

invagination of the basolateral membrane encircling leukocytes (Fig. 2.4A-D). Many cells in this region were distinct from the surrounding epithelium and displayed characteristics typical of bovine M-cells (Liebler, Pohlenz, and Woode, 1988;Torres-Medina, 1981). The epithelium adjacent to FAE also showed heterogeneity, interspersed with M-cells but predominated by an array of polygonal epithelial cells with regular, long, uniform and densely-packed microvilli typical of enterocytes. Epithelium not associated with follicles showed an homogenous enterocyte population with long uniform microvilli.

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2.3.4 Lectin binding by FAE

Although various lectins have assisted differentiaton of FAE in other animals (Table 2.2) none of the lectins used in this study specifically labelled FAE of the bovine terminal rectum. Each lectin was tested at a range of dilutions on tissue fixed with three different methods (paraformaldehyde, ZSF or cryo-fixation). Depending upon concentration, the lectins either did not label epithelium at all or labelled all epithelial cell types with similar intensity. This staining pattern was consistent with that from on-going investigations of sheep intestinal tissue (J Huntley, unpublished observations) using a panel of 30 lectins (also including UEA, consistently specific for mouse and rabbit M-cells) and hence is not unique to cattle but reflects the absence of a consistent glycoconjugate to differentiate M-cells and highlights the crucial need for such M-cell markers.

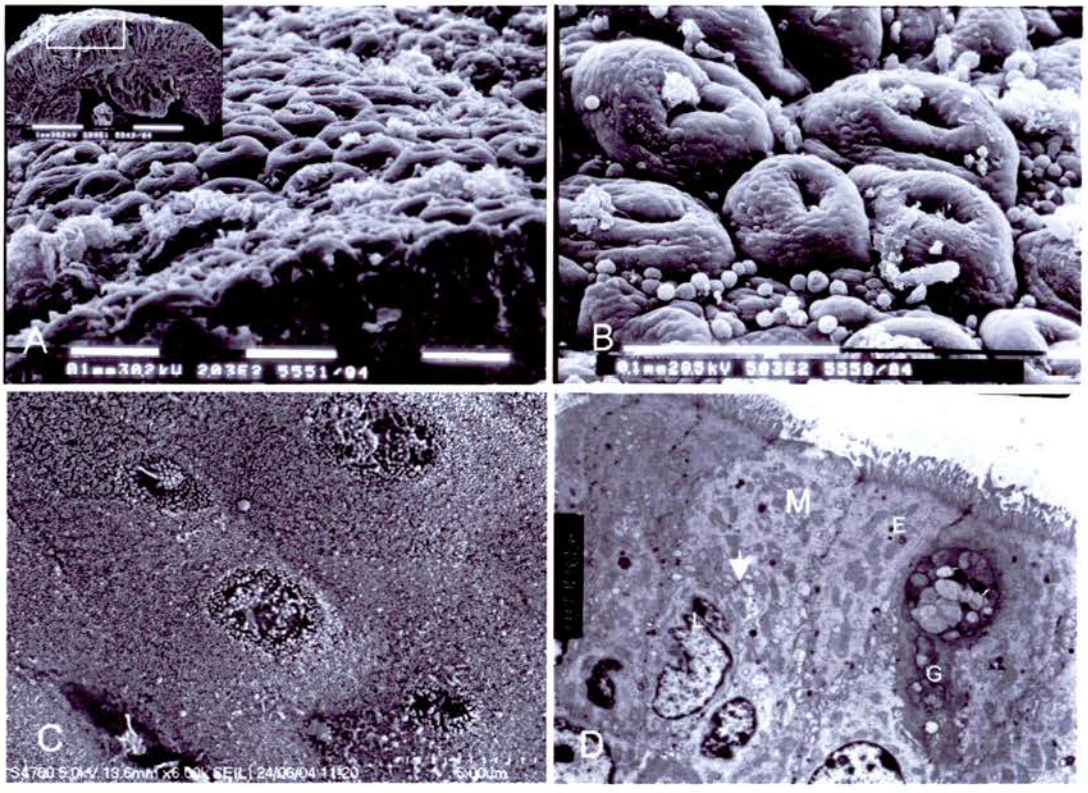

Fig. 2.4 Ultrastructure of follicle associated epithelium in the bovine terminal rectal mucosa.
A: Low magnification (x250) SEM image showing the lumenal surface of follicle-associated crypts. The inset (35x magnification) shows the region within the follicle-associated tissue from which the higher magnification images were taken.
B&C: Higher magnification (x600 and x6000 respectively) SEM images of crypts showing heterogeneous cell populations, predominantly expressing short microvilli – M-like cells.
D: TEM showing M-cell with stunted or fused microvilli with a leukocyte (L) in an intra-epithelial pocket on the basolateral surface. Adjacent enterocyte (E) and goblet (G) cells are also visible (2950x magnification).

2.3.5 Microparticle Uptake by FAE

To further characterise the cells in this region an *in vitro* and *in vivo* functional assay specific for the transcytosis activity of M-cells was performed. Tissue explants comprising FAE from the regions 0-3 cm or 18-20 cm cranial to the RAJ were cultured as described in "Materials and Methods". Fluorescent latex beads incubated with explants for 45 minutes - during this period tissue explants retained an intact, functional mucosal epithelium as observed herein and in previous investigations (Jackson *et al.*, 2004). Fluorescent confocal microscopy revealed microparticles present on the apical surface of FAE (Fig. 2.5A) whereas microparticles could not be detected in association with the absorptive epithelium either adjacent to FAE or from mucosa 20 cm proximal to anal-rectal junction. This finding is consistent with localisation of M-cells to FAE as shown by immunohistochemical staining for vimentin as described above. Uptake of microparticles by some cells within the FAE (Fig. 2.5B) was also demonstrated by confocal microscopy – optical sectioning of cells showed fluorescent particles subjacent to the apical actin cytoskeleton (stained with phalloidin). There was no apparent difference in the cell association and uptake of particles of different sizes (0.2 μm and 0.5 μm). *In vivo*, particle uptake from the rectal lumen was evident from 45 minutes post-challenge and was restricted to the dome epithelium (Fig. 2.5C) of the rectum. At the later time point (60 minutes) beads were observed within the submucosal region (Fig. 2.5D-F), indicative of microparticle transcytosis.

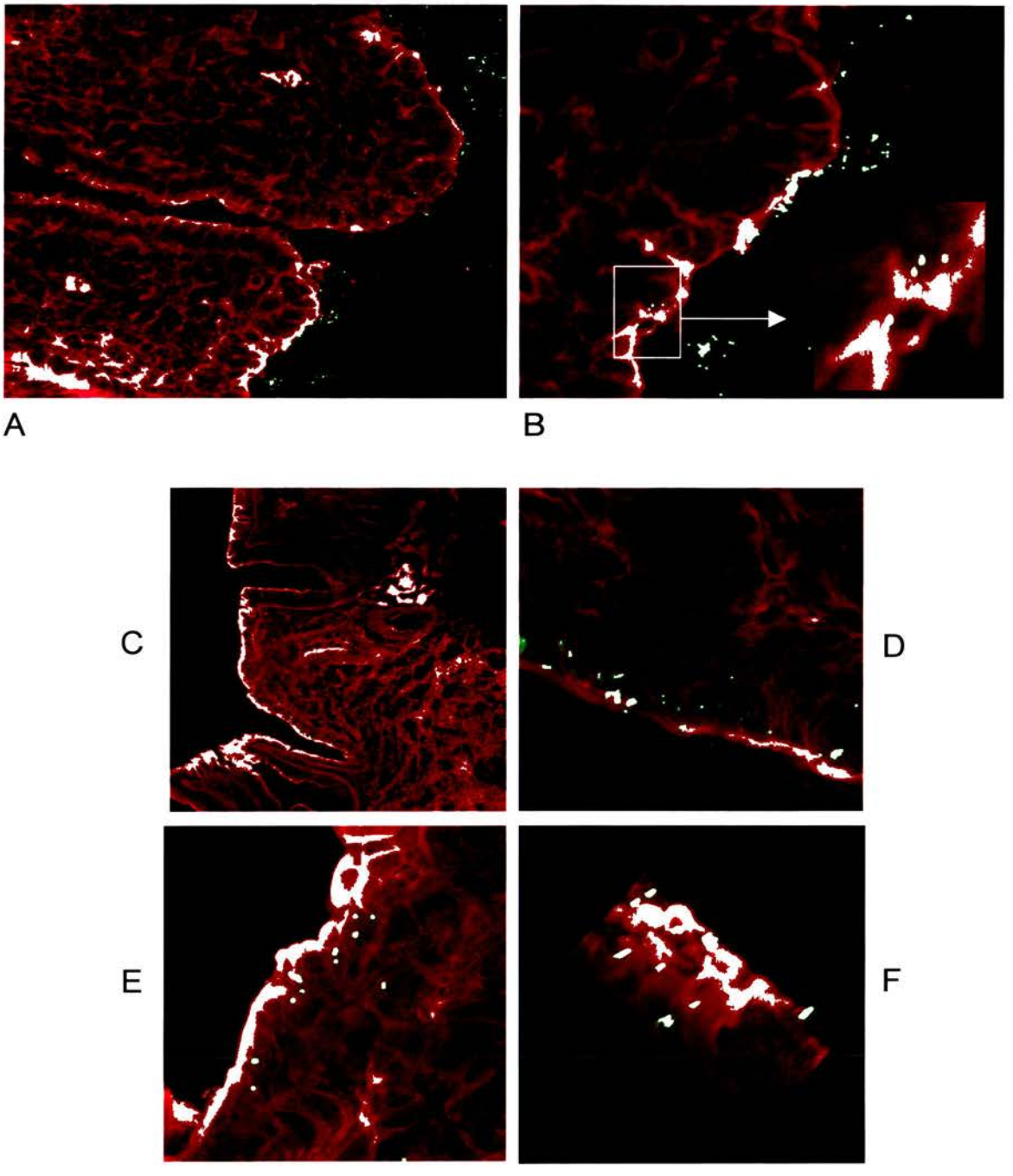

Fig. 2.5 Microparticle uptake by rectal tissue explants (A&B) or rectal FAE *in situ* in calves (C,D&E). Cryosections demonstrate adsorption and transport of 0.5μm fluorescent latex microspheres (green). Tissues were counter-stained with Phalloidin-TRITC (red) to visualise cellular cytoskeleton. These images show tissue samples from approx. 45minutes post-challenge and are representative of results at 45-60 minutes post-challenge. Microspheres adhered to and were taken up by a subset cells in the FAE, a characteristic of M-cells. Images A&C were acquired with 20x and images B&D with 40x and 63x lens, respectively. The inset in Fig.2.5B is a higher magnification (63x, zoom factor 2). Images E&F shows particle internalisation. For this, a series of optical sections were acquired on the Leica TCS NT confocal system. These sections were then visualised in the Surpass module of the Imaris software suite (Bitplane AG, Zurich), which converts the image voxels into a geometric object. Following threshold segmentation, the image was then surface rendered, allowing volumes and interactions between objects to be defined.

2.4 Discussion

Lymphoid follicles (LF) and the overlying follicle-associated epithelium (FAE) form discernable structures in the intestinal mucosa with important functions in generating immune responses to mucosal pathogens. In the current investigation, lymphoid follicles clustered at high density in the region 0-3 cm proximal to anal-rectal junction (RAJ) of cattle forming compact clusters whereas in more proximal sites in the rectum there were solitary, discrete follicles, becoming inapparent at more proximal sites. Similar to our findings, distribution of patches of lymphoid tissue around the circumference of the anal canal have been observed in other mammals including humans (Gebbers, Kennel, and Laissue, 1992) and sheep (Sedgmen *et al.*, 2002) with the lymphoid follicles more frequently present in the submucosa than the lamina propria as observed herein. Therefore, rectal lymphoid tissues are similar in several animal species therefore this investigation focussed on cattle as representative mammal in which to characterise the cell populations within this site to assess the potential of this tissue as an immune inductive site.

The immune inductive role is dependent upon the presence and function of cell populations necessary for antigen sampling, processing and induction of specific immune responses including M-cells, APCs and B- and T-lymphocytes. Immunophenotyping of lymphoid follicles underlying rectal FAE showed distinct T-lymphocyte (CD4+, CD8+, γδTCR+), B-lymphocyte (CD40, CD21, CD25), dendritic cell (CD1b) and IgA/IgM staining patterns were observed within and around follicles. Thus rectal lymphoid follicles resemble LFs from other sites (Sedgmen *et al.*, 2002;Nagi and Babiuk, 1987;Gutierrez *et al.*, 1999), with APC and lymphocyte populations necessary for development of adaptive immune responses. Specialised epithelial cells involved in antigen sampling normally overlie lymphoid follicles and these cells differ from enterocytes in morphology, function and other characteristics. Ultrastructural examination of the dome epithelium overlying follicles revealed that follicle-associated epithelium (FAE) consisted of more than one cell type. Numerous cells with short and sparse microvilli (indicative of M-cells) were observed adjacent to cells showing uniform, long microvilli (indicative of enteroabsorptive cells). FAE had no, or only occasional, goblet cells although this

cell type became evident in the transitional region from FAE to mucosal villous epithelium. M-cells represent a specialised epithelial cell population which samples lumenal macromolecular and particulate antigens for delivery to underlying antigen processing cells (APCs) in lymphoid follicles (Owen, 1999).

Specific glycoconjugates have been widely used to mark the apical membrane of M-cells (Gebert and Posselt, 1997;Giannasca *et al.*, 1999;Gebert and Hach, 1993a;Clark *et al.*, 1993). However, with the exception of rabbit, mouse and other selected examples (see Table 2.2), no lectin has been identified that can be used as a common marker for these cells from different intestinal regions or in different animal species. The present investigation supports this conclusion since none of the lectins exhibited specificity for either M-cell or the FAE in the terminal rectum of cattle, an observation consistent with FAE of sheep (J Huntley, unpublished) with which a panel of thirty lectins also failed to identify FAE/M-cell specific glycoconjugates. A great diversity of expression of M-cell-specific glycoconjugates has been observed both between animals and tissues (for example, UEA-1 is a marker for M-cells in Peyer's patches but not in the colon (Giannasca *et al.*, 1994) of mice). Glycoconjugates characteristic of, and sufficient to differentiate, cattle (or sheep) FAE or M-cells still remain to be defined and the current study highlights the crucial need for consistent M-cell markers to aid in further characteriation of M-cell biology.

M-cells also express characteristic intermediate filaments which are absent in the absorptive enterocytes. Vimentin and cytokeratin 8 and 18 are found in rabbit, rat and pig intestinal M-cells (Jepson *et al.*, 1992;Gebert, Hach, and Bartels, 1992;Rautenberg *et al.*, 1996) but absent in enterocytes. In this study, a strong staining for vimentin in the majority of cells of the FAE in the rectal mucosa and most of the epithelial cells in crypts in close proximity to lymphoid follicles was observed. This staining pattern in dome-associated crypts resembles that observed previously in rabbits (Lelouard *et al.*, 2001a) and these cells may be undifferentiated or precursor M-cells in the crypts adjacent to FAE (Gebert *et al.*, 1999). Vimentin staining was absent in epithelium not associated with follicles, such as the mucosa 20 cm proximal to the recto-anal junction.

Although the considerable variation in the histochemistry of M-cells makes it difficult to characterise these cells fully, they can be defined by their key function i.e. particulate/macromolecular antigen uptake and transcytosis, a characteristic which has been defined both in vivo and in vitro using microparticle uptake assays (Porta *et al.*, 1992b;Ermak *et al.*, 1995;Pappo and Ermak, 1989;Landsverk, 1988). In this study, functional assessment of M-cells was carried out in tissue explants from terminal rectal mucosa and by in vivo calf challenges. Both 0.2 μm and 0.5 μm microparticles adhered to, and were internalised by, cells in the FAE. Other epithelial cells, within both rectal FAE and mucosa 20 cm proximal to anal-rectal junction, did not show microparticle attachment and uptake activity. The localisation of this function to rectal FAE correlates with the immunohistochemical detection of vimentin-expressing cells, thus supporting the conclusion that these cells do indeed represent M-cells and are involved in antigen sampling and delivery to rectal lymphoid follicles for induction of specific immune responses via rectal LF.

The finding that not all cells of FAE bound and internalised microparticles, although the majority of these cells were vimentin-positive, was intriguing and of possible functional significance. The ontogeny of M-cells remains a matter of much debate and whether the subsets of vimentin-expressing cells in the rectal epithelium represent different stages in M-cell development or even different lineages remains to be defined as does their involvement in immune response induction. This study did not aim to directly address whether M-cells represent a distinct lineage or an additional epithelial cell phenotype however it can be concluded that juxtaposition of epithelium and lymphoid follicles is an important determinant in triggering M-cell phenotype in both FAE and adjacent, follicle-associated crypts. Lymphotoxin has been suggested as one possible effector (Debard *et al.*, 2001) although much further study will be required to elucidate the signals responsible for regulating M-cell development.

This investigation has demonstrated that FAE overlying lymphoid follicles in terminal rectum was predominated by cells expressing vimentin, a feature of M-cells.

A subset of these cells bound and internalised microparticles, a key characteristic of M-cells. Importantly, within lymphoid follicles of this site antigen presenting cells and lymphocytes were present, both cell types that are necessary for development of specific immune responses. Although additional investigation will be required to further characterise FAE cell subsets, cellular determinants involved in FAE interactions with pathogens and the role of this site in development of immune responses, the current investigation has demonstrated that terminal rectum possesses the cellular populations characteristic of mucosal immune inductive sites and may thus play a role in development of mucosal immune responses. These findings will also be helpful to understand the biology of *E. coli* O157:H7 interaction at the terminal rectum, the principal site of its colonisation in cattle.

3 Development and characterisation of Primary epithelial cell culture from bovine terminal rectal mucosa

3.1 Introduction

Primary epithelial cell culture from the terminal rectum, the primary site of *E. coli* O157:H7 colonization in cattle, was developed as an in vitro model to examine the adherence and functional interactions of EHEC. Different cell lines, primarily Hep-2 (human laryngeal epidermoid carcinoma) and HeLa (cervical carcinoma) cells have been used extensively to examine adherence mechanisms of EPEC and EHEC. A/E lesion are readily formed by LEE-positive strains on these cells and the fluorescent actin staining (FAS) test has been developed to provide a relatively simple assay for the A/E phenotype on such cells (Knutton *et al.*, 1989). Numerous discoveries relating to the mechanism of A/E lesion formation have been made using these cells lines and the role of intimin in the adherence of *E. coli* O157:H7 has been expanded by the observation that a cell surface molecule of Hep-2 cells, nucleolin (Sinclair and O'Brien, 2002), co-localises with intimin. T84 and Caco-2 human colonic carcinoma cells have advantages over the previous cell lines in that they polarize and form tight junctions on collagen-coated surfaces, producing a monolayer that is physically more like natural epithelium. These two cell lines differ in Gb3 expression and hence are good models to study the role of VT in EHEC interaction with intestinal epithelium. Caco-2 cells express Gb3 and are described as being villous-like, whereas T84 cells lack Gb3 and resemble intestinal crypt cells. The T84 cell types have extensively been used to model human intestinal epithelial cells which lack Gb3 *in vivo* (Holgersson, Jovall, and Breimer, 1991). Although the cell lines have the convenience of being sub-culturable indefinitely, there is no guarantee that these cell lines express cellular receptors that occur *in vivo*. Alternatively, primary epithelial cell cultures which resemble native intestinal epithelium in many important characteristics, provide a relevant system to examine EHEC interaction with and effects of VT (or EHEC factors) on the bovine host. This has been used in one study (Dibb-Fuller *et al.*, 2001) in which *E. coli* O157:H7 formed A/E lesions on bovine primary epithelium and showed adherence levels higher than EPEC O111 and *E. coli* K12. In another study bovine colon epithelial cells expressed Gb3 and localized VT

to the lysosomal compartments leading to abrogation of cytotoxicity (Hoey *et al.*, 2003a).

Intestinal epithelium is composed of multiple cell types including absorptive enterocytes, enteroendocrine, goblet and Paneth cells. These cells are derived through asymmetrical division, migration and differentiation from pluripotent stem cells. An additional specialised epithelial cell type, termed M-cells ("membranous" or "microfold" cells), are associated particularly with epithelium overlying the gut-associated lymphoid tissue. This is referred to as follicle-associated epithelium (FAE) and is a site of active immunological function. In contrast to villous epithelium, FAE contains no or fewer goblet cells (Owen, 1999), defensin- and lysozyme-producing Paneth cells (Giannasca *et al.*, 1994) and expresses low amounts of membrane-associated hydrolases (Owen and Bhalla, 1983). The M-cells generally lack the distinct microvilli and thick filamentous brush border glycocalyx (Frey *et al.*, 1996b) and instead have variable microfolds. Together, these features of M-cells promote contact of antigens with gut epithelium and result in sampling of antigens from the intestinal lumen and transfer to antigen presenting cells (APCs) within an intra-epithelial pocket (Neutra, Pringault, and Kraehenbuhl, 1996) on its basolateral side (Ermak, Bhagat, and Pappo, 1994;Farstad *et al.*, 1994;Iwasaki and Kelsall, 2001). At the interface of luminal microflora and the mucosal-associated lymphoid tissue (MALT), the M-cells play a crucial role in regulating the access of microorganisms/antigens and the generation of protective immune responses.

Like villous epithelium, FAE is derived from the crypt stem cells. Each crypt is a clonal unit of pluripotent stem cells anchored together as a ring which give rise to multiple cell types that migrate upward in columns onto several adjacent villi (Gordon and Hermiston, 1994;Cheng and Leblond, 1974;Schmidt, Wilkinson, and Ponder, 1985). FAE is formed by convergence of migrating cells from 12 or more follicle-associated crypts (Gebert, Hach, and Bartels, 1992;Savidge and Smith, 1995;Bye, Allan, and Trier, 1984). The follicle-associated crypts have two distinct axes of migration/differentiation. Cells located on one side of the crypt migrate and differentiate into villous epithelium with absorptive enterocytes, goblet and

enteroendocrine cells whereas cells on the follicle side of the crypt move onto the dome and differentiate into FAE and M-cells (Kraehenbuhl and Neutra, 2000;Gebert *et al.*, 1999;Bye, Allan, and Trier, 1984). There are various hypotheses regarding the ontogeny of FAE and M-cells: (i) M-cells may originate in the crypts as a distinct cell lineage from specific progenitor stem cells via an independent differentiation programme (Gebert and Posselt, 1997;Gebert *et al.*, 1999;Bye, Allan, and Trier, 1984); (ii) they may derive from enterocytes under the influence of surrounding lymphoid micro-environment (Kerneis *et al.*, 1997;Savidge, 1996;Smith and Peacock, 1980); (iii) exposure to microbial pathogens in the lumen may induce enterocytes to specialise into M-cells (Meynell *et al.*, 1999;Savidge *et al.*, 1991;Borghesi *et al.*, 1996;Borghesi, Taussig, and Nicoletti, 1999); or (iv) they represent a transient phenotype of FAE enterocytes as they migrate from the crypts to the apex of the dome (Sierro *et al.*, 2000).

Whichever hypothesis/es is/are correct, M-cells represent an important interface between immunogens in the gut lumen and the immune system. This is an intimate relationship as evidenced by work showing that FAE secrete certain homing chemokines that are involved in function and maintenance of organised MALT (Iwasaki and Kelsall, 2000).

Despite their importance, studies of the M-cells are very limited due to the lack of any M-cell line or a system to readily reproduce these cells in vitro. This chapter describes a primary culture protocol which produces cells with phenotypic and functional characteristics of M-cells. It provides a suitable *in vitro* model to study the interaction with *E. coli* O157 H7 which principally colonises the M-cell/FAE rich lymphoid-follicle dense mucosa at the terminal rectum of cattle.

3.2 Materials and Methods

3.2.1 Reagents used in the primary cell culture

High (4.5 g/l) glucose 1X DMEM, MEM D-Val, HBSS and Amphotericin B were obtained from GIBCO BRL (Paisley, Scotland); and Dispase I (neutral protease) were obtained from Roche Diagnostics Gmbh (Mannheim Germany). Insulin,

Epidermal Growth Factor (EGF), Penicillin-Streptomycin solution, Collagen Type IV, Collagenase and Gentamicin were purchased from Sigma, (Irvine, UK). Tissue culture 4 well Permanox Plastic slides and Thermonox coverlips were used for primary epithelial cell culture (LAB TEK Nalge Nunc International). FITC labelled VT1 B-subunit was kindly provided by Prof. Clifford A. Lingwood, Toronto, Canada.

3.2.2 Specimen preparation

The terminal rectum with intact anus from adult cattle was obtained from a local abattoir. Immediately upon excision the specimens were placed in HBSS containing gentamicin (25 μg/ml) on ice and transported to the laboratory within 30 minutes. The specimens were opened longitudinally and the luminal contents flushed out with HBSS. Two pieces of mucosal epithelium 3 cm and 25 cm proximal to recto-anal junction were excised and washed vigorously several times in HBSS supplemented with gentamicin (25 μg/ml), penicillin (100 U/ml), streptomycin (30 μg/ml) and amphotericin B (200 μg/ml) in a sterilized tissue collecting pot to remove mucus and any adhering contaminants/materials. Tissue was then processed as below to isolate crypts.

3.2.3 Crypt isolation procedure

The procedure for primary cell culture was essentially as described by (Hoey *et al.*, 2003a;Booth *et al.*, 1995) with minor modifications. The mucosal epithelium was scraped with a sterile glass slide and the scrapings collected into a 50 ml centrifuge tube. The scrapings were given three washings in HBSS to remove the cell debris and mucus. The pelleted tissue was digested in 25 ml complete DMEM (1% FCS, 100 U/ml penicillin, 30 μg/ml streptomycin, 25 μg/ml gentamicin) containing 75 U/ml collegenase and 20 μg/ml dispase for 80 minutes with gentle shaking at 37°C. The digested tissue was gently vortexed to release the crypts. An aliquot of digested tissue suspension was observed microscopically to confirm the yield and integrity of the crypts. To isolate the crypt, the suspension was centrifuged 5 times at 1200 RPM for 2 minutes on a 2% D-sorbitol gradient. The isolated crypts were washed in HBSS, gently suspended in primary culture media (DMEM media supplemented with 1% or 2.5% batch-tested FCS, 0.25 U/ml insulin, 10 ng/ml EGF and 30 μg/ml

gentamicin) and plated into four well tissue culture chamber slides coated with Collagen Type IV. For collagen coating, 400 µl of 200 µg/ml acidified collagen was added to each well and left overnight to air dry in a sterilized laminar flow hood.

3.2.4 Cell culture

The isolated crypts were suspended in 10 ml of primary culture medium and 100µl of the suspension was used to count the number of crypts in the suspension. Approximately 500 crypts were added to each well of 4 well-Permanox plastic slides. The crypts were cultured in primary culture medium for 24 h. At this stage most of the crypts had attached to the surface, the non-adherent tissue debris was removed and media was replaced with MEM D-Val selective medium supplemented 1% or 2.5% batch tested FCS, 0.25 U/ml insulin, 10 ng/ml EGF and 30 µg/ml gentamicin. This medium inhibits any fibroblast contaminants in the culture (Hoey *et al.*, 2003b;Frauli and Ludwig, 1987;Lazzaro *et al.*, 1992). The cultures were fed two days at which times 50% of the medium was replaced. The epithelial cells proliferated around attached crypts and formed a confluent monolayer within 6 to 8 days of culture. The cultures were incubated at 37°C in an atmosphere of 5% CO_2, 95% air with 90% relative humidity.

3.2.5 Characterisation of the Bovine Rectal Primary Epithelial Cells

The epithelial origin of the cells was confirmed by immunostaining for cytokeratin intermediate filaments. The cells at five days of culture were fixed with 2% paraformaldehyde (PFA), permeabilized with cold acetone for 5 minutes, washed with phosphate-buffered saline (PBS) and stained with a pan-cytokeratin monoclonal antibody (Sigma 1:300) for 3 h at 25°C. The cells were washed with PBS and the monoclonal was detected using FITC-labelled goat anti-mouse mAb (1:80) (Sigma). The cell nuclei were stained with TO-PRO Iodide (Molecular Probes). The stained cells were mounted in fluorescence mounting medium (Fluoromount, DAKO) and examined using a Leica DMLB epifluoresence microscope.

The secretory function of cells was demonstrated by staining the type of mucins secreted as previously described (Chen *et al.*, 1993;Latella *et al.*, 1996). Briefly, the cells were fixed in Bouin solution (saturated aqueous picric acid, 40% formaldehyde

and glacial acetic acid 15:5:1 v/v/v) and stained with Periodic Acid Schiff (PAS) and Alcian Blue (AB) at pH 1.0.

To determine areas of cell proliferation in the culture, staining for the proliferating cell nuclear antigen (PCNA) was done as described previously (Hoey *et al.*, 2003b). The bovine primary rectal epithelial cells at 96 h of culture were fixed and permeabilized in 2% PFA and cold methanol, respectively for 5 minutes each. The cells were washed in PBS and stained with R-phycoerythrin–conjugated mouse anti-PCNA (1:100) monoclonal for 30 minutes at 37°C. After further washing the cells were mounted in Fluoromount and examined using a Leica TCS NT confocal microscope (Leica Microsystems, Heidelburg).

To localise Gb3 receptor the bovine rectal primary epithelilial cells were incubated with FITC-labelled VT1 B-subunit (0.5 mg in 100 ml of 2% BSA in PBS) for 1 h in the dark at 4°C to prevent toxin internalization. After gentle washing with ice-cold PBS, the cells were fixed with 2% PFA and the slides were mounted in Fluoromount and examined using a Leica DMLB epifluorescence microscope.

For ultrastructural analysis, the cultured cells were prepared for transmission and scanning electron microscopy as previously described (Latella *et al.*, 1996). Briefly, the isolated rectal crypts were seeded on collagen-coated 13 mm Thermanox coverslips and 6 day-old cells in culture were fixed with 3% gluteraldehyde in 0.1% sodium cacodylate buffer at pH 7.4 for 2 h at 4°C. The samples were rinsed with the same buffer and post-fixed for 2 h with 2% osmium tetroxide in 0.1M cacodylate. The samples were dehydrated through a series of graded alcohols, dried in a critical point drying system and sputter coated with 20 nm gold/palladium (60/40) (Emscope SC 500 Sputter Coater). The samples were then examined in a scanning electron microscope (Philips 505). For transmission electron microscopy the cells were fixed in 3% gluteraldehyde in 0.1% cacodylate buffer at pH 7.4 for 2 h postfixed in 1% OsO_4 for 30 minutes, dehydrated through a series of graded alcohols and embedded in Epon/Araldite mix. Ultrathin sections (80 nm) were cut and counterstained in 2%

uranyl acetate and lead citrate and observed in the Phillips CM12 Transmisssion microscope.

3.2.6 Screening of primary epithelial cell monolayer for immune cells

To ascertain whether lymphoid cells were present in primary cell cultures from the crypts isolated from lymphoid-rich mucosal tissue, immunostaining was done with a panel of seven monoclonal antibodies (Table 3.1), specific for different immune cell types of cattle. The cells were fixed either in paraformaldehyde (3.5%) or non-formaldehyde zinc acetate based fixative (adapted from (Gonzalez *et al.*, 2001). Briefly, cells (at 72 h of culture) were washed twice in PBS and fixed in freshly prepared zinc salt fixative solution (0.1M Tris base buffer with Ca acetate 0.5% pH 7-7.4, containing Zn acetate 0.5% and Zn chloride 0.5%). After initial blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide for 5 minutes and the non-specific binding with 25% normal goat serum for 30 minutes the cells were immunolabelled overnight at 4°C (Table 2.1). The primary antibody binding was detected using goat anti-mouse peroxidase labelled secondary antibody (Horizontal En Vision Plus HRP system, Dako, Ely, UK) for 30 minutes at RT. The slides were developed in a citrate buffer containing 3,3' diaminobenzidine hydrochloride for 7-8 minutes. Cell monolayers were counterstained with haematoxylin, rinsed, dehydrated and mounted in DPX (BDH Laboratory Supplies Poole, UK). Tris-buffered saline (TBS, 0.05 M Tris HCl, 0.15 M NaCl, pH 7.6) was used to wash the slides between each stage of the labelling procedure and to dilute normal goat serum and antibodies. As a negative control primary antibody was replaced with TBS in the staining protocol.

3.2.7 Microparticle uptake assay and the co-localisation studies

Uptake of inert microparticles has been used as a functional assay for M-cells in *in vitro* cultures (Kerneis *et al.*, 1997). To assess if the cells in primary culture were capable of taking up beads, this assay was performed with latex fluorescent particles. FITC-conjugated latex beads of 0.5 μm size (Polysciences Inc., Germany) were diluted (1:1000) in DMEM containing 2% fetal bovine serum. Aliquots (100 μl) of diluted beads were pipetted evenly on to 6-day old epithelial monolayer and incubated at 37°C for 45 minutes. The cells were washed three times in phosphate-buffered saline, fixed and permeabilized with 2% (w/v) PFA/ 0.25% (v/v) Triton X-100 at room temperature for 20 minutes. Staining of F-actin was performed with Phalloidin-FITC/TRITC (diluted 1:20 in PBS; Molecular Probes) for 45 minutes at room temperature in the dark. For colocalization studies with vimentin, the cells were incubated with mouse anti-vimentin monoclonal antibody (1:100) (Sigma) and incubated overnight at 4°C. This primary antibody was detected with Alexa 594-tagged rabbit anti-mouse or goat anti-rabbit monoclonal antibodies as per the manufacturer's instructions. The cell nuclei were stained with TO-PRO Iodide (Molecular Probes). The slides mounted in fluorescence mounting medium (DAKO) were examined using a Leica TCS NT confocal microscope (Leica Microsystems, Heidelburg). To examine the position of the beads, 0.4 μm optical sections were acquired and processed via Imaris Surpuss Module (Bitplane) computer software programme.

3.2.8 Adherence assay and co-localisation studies

To assess if the cells in primary culture, expressing vimentin or capable of taking up beads, were also able to bind bacteria, adherence assays with *E. coli* O157:H7 strains followed by counterstaining with vimentin was done.

E. coli O157:H7 strains (ZAP 198-GFP/ pACYC184 and ZAP 198-RFP/pBAD) were kindly provided by Dr. Andy Roe and Dr. Alison Low, ZAP Lab. The overnight cultures of bacterial strains in M9 medium (Sigma) with ampicillin/chloramphenicol (25 μg/ml) were used to inoculate 20 ml of fresh media. The strains were induced with Arabinose (0.2% v/v) and incubated for approximately 3h at 37°C, 200 rpm in a shaking incubator to an optical density of 0.3-0.4 at OD_{600}. An aliquot (100μl) of

bacterial culture (approximately $3x10^8$ cfu/ml) was used to infect confluent bovine rectal primary epithelial cells, in duplicate, at a multiplicity of infection (MOI) 1:100 in MEM-HEPES. The primary epithelial cells, prior to infection, were washed twice and incubated in 400 μl of pre-warmed (37°C) MEM-HEPES medium. The cells were infected for 1h at 37°C in a 5% CO_2 atmosphere. Non-adherent bacteria were removed by washing three times with phosphate-buffered saline (PBS), and cells were fixed/permeabilized in 4% (w/v) paraformaldehyde (PFA)/0.2% (v/v) in PBS for 20 minutes at room temperature. For colocalisation studies, the fixed/ permeabilized cells were incubated with mouse anti-vimentin monoclonal antibody (1:100) (Sigma) and processed for immunofluorescence studies as described above.

For colocalisation studies with microparticles the infected cells were washed three times with prewarmed (37°C) MEM/HEPES and further incubated with FITC-conjugated latex beads of 0.5 μm size (Polysciences Inc., Germany) for 45 minutes and processed for immunofluorescene studies as described above.

3.3 Results

The use of enzymatic digestion, with gentle mechanical agitation, yielded intact and viable crypts (Fig. 3.1A) from bovine rectal mucosa. The subsequent differential centrifugation in 2% D-sorbitol gradient eliminated damaged crypt cells and contaminating stromal cells, most of which were fibroblasts. The few remaining fibroblasts were eliminted using MEM D-Val selection media with only 1% FCS, conditions that favour growth of epithelial cells over fibroblasts which cannot survive in this L-Valine-deficient medium (Hoey *et al.*, 2003b;Frauli and Ludwig, 1987;Lazzaro *et al.*, 1992). The absence of fibroblasts in the culture on the 4th day was further checked using fibroblast specific monoclonal antibody (hPH) that recognises prolyl 4-hydroxylase required in the synthesis of collagen.

About 20% of crypts attached to the collagen-coated surface after 24 h and were then cultured on selective media when the non-adherent debris was removed. At this stage a cohort of epithelial cells could be seen growing outwards from crypts (Fig. 3.1B).

Proliferating epithelial cells, marked by staining for the proliferating cell nuclear antigen (PCNA) (Fig. 3.2B), emerging from adjacent crypts formed a confluent patch of epithelium (Fig. 3.1C). After 6 days of culture a compact epithelial monolayer with typical cobblestone morphology was obtained (Fig. 3.1D).

Cultured cells expressed the epithelial cell-specific cytokeratins (CK4, 5, 6, 8, 10, 13 and 18). In Fig. 3.2A columns of cells expressing cytokeratins can be seen emerging from the crypt. The secretory function of cultured primary epithelial cells was demonstrated by mucin cytochemistry using PAS and AB stainings. After 3 days of culture about 1/3rd of the cells were PAS-positive while AB staining was restricted to a very small proportion of cells (<5%). Dual staining with PAS and AB demonstrated only a very few cells positive (<1%). Ultrastructural studies by SEM and TEM demonstrated the differentiated status of cultured cells in particular cells with polarized morphology, apical microvilli and tight junctions between neighbouring cells. Occasional goblet cells were also observed in the monolayer (Fig. 3.3A, B, C). Some of the cells expressed GB3 (Fig. 3.3 D).

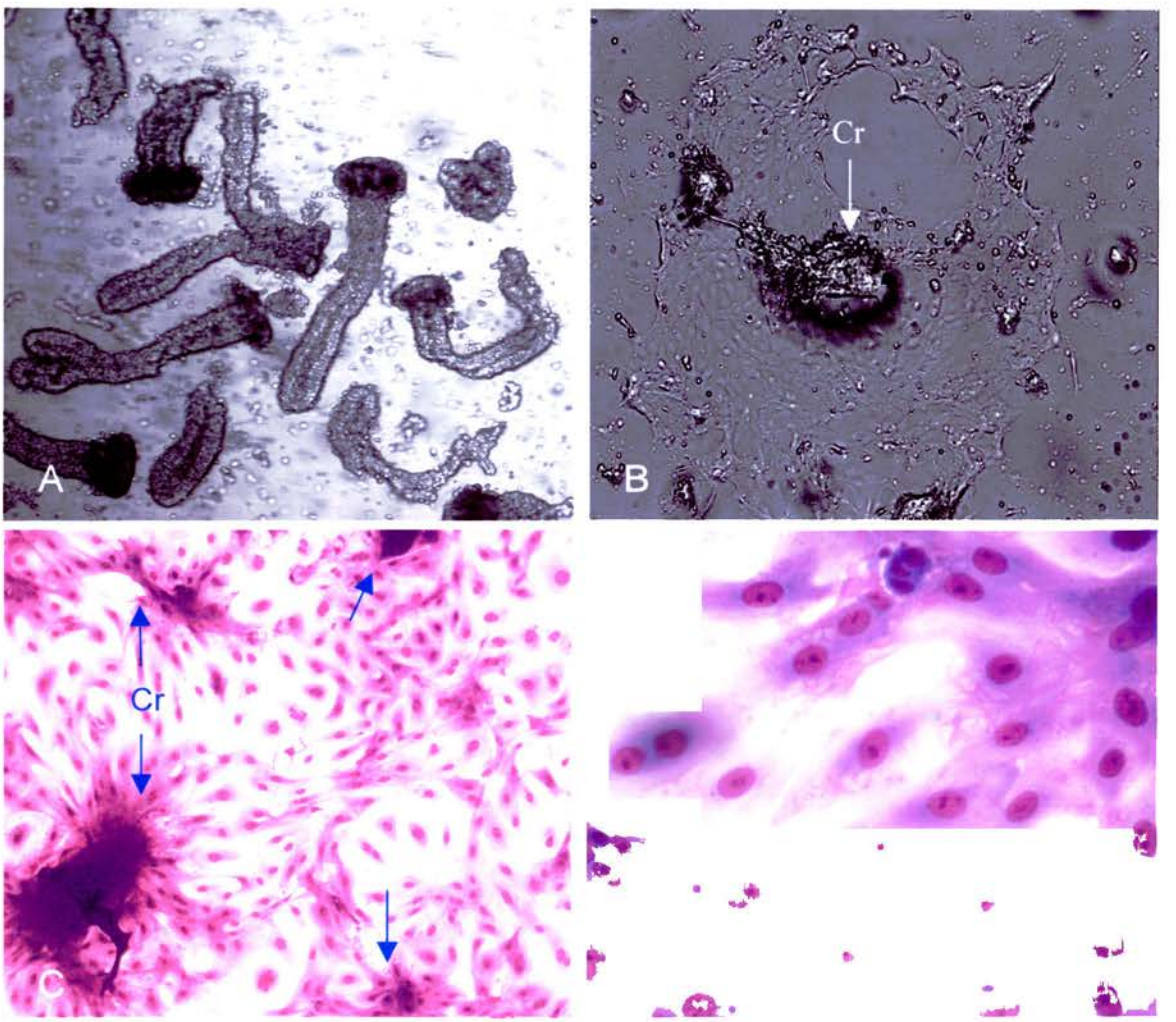

Fig. 3.1 Primary cell culture growth. (A) Bovine rectal crypts released on digestion with collagenase and dispase enzymes, enriched on 2% D-sorbitol gradient. (B) A colony of epithelial cells emerging from a single crypt (Cr) attached to the collagen-coated surface 24 hours of culture. (C) Epithelial monolayer (stained with Diff-Quik) formed on convergence of migratory colonies of epithelial cells from the adjacent crypts post 72 h of culture. (D) A compact monolayer of epithelial cells with a typical cobblestone morphology on 7 day of culture.

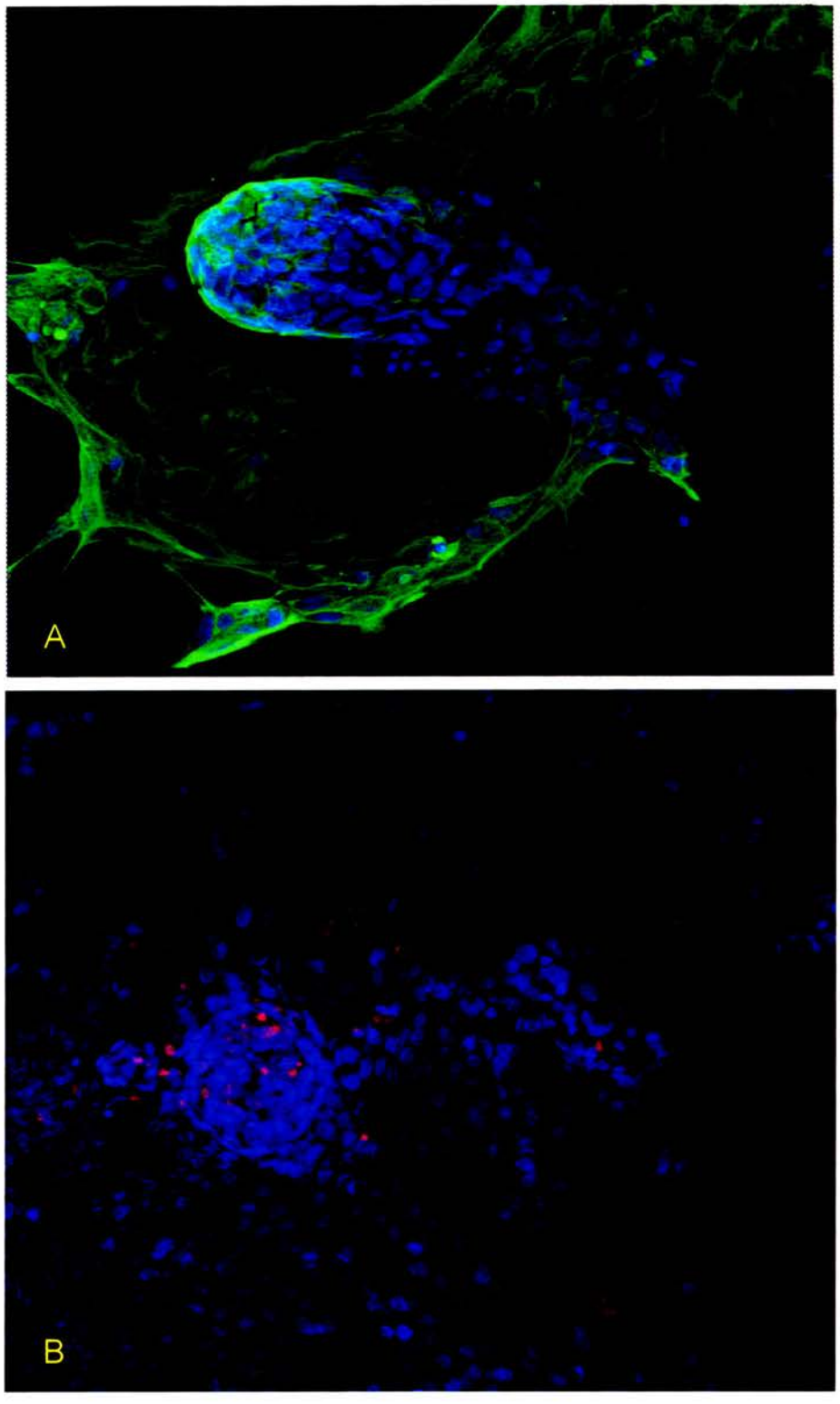

Fig. 3.2 Phenotyping of primary cell culture. (A) Columns of cells emerged from a bovine rectal crypt, immunolabelled with anti-pancytokeratin (green) antibody and nuclear stain TO-PRO (blue). All the cells in the colony expressed epithelial specific cytokeratins. (B) A subset of cells in the crypt unit and adjacent epithelial monolayer stained positive for PCNA-RPE (red), a proliferation marker; but the majority of the cells in the monolayer were negative for this protein. Images were acquired using Leica TCS NT confocal system (x63 objective lens).

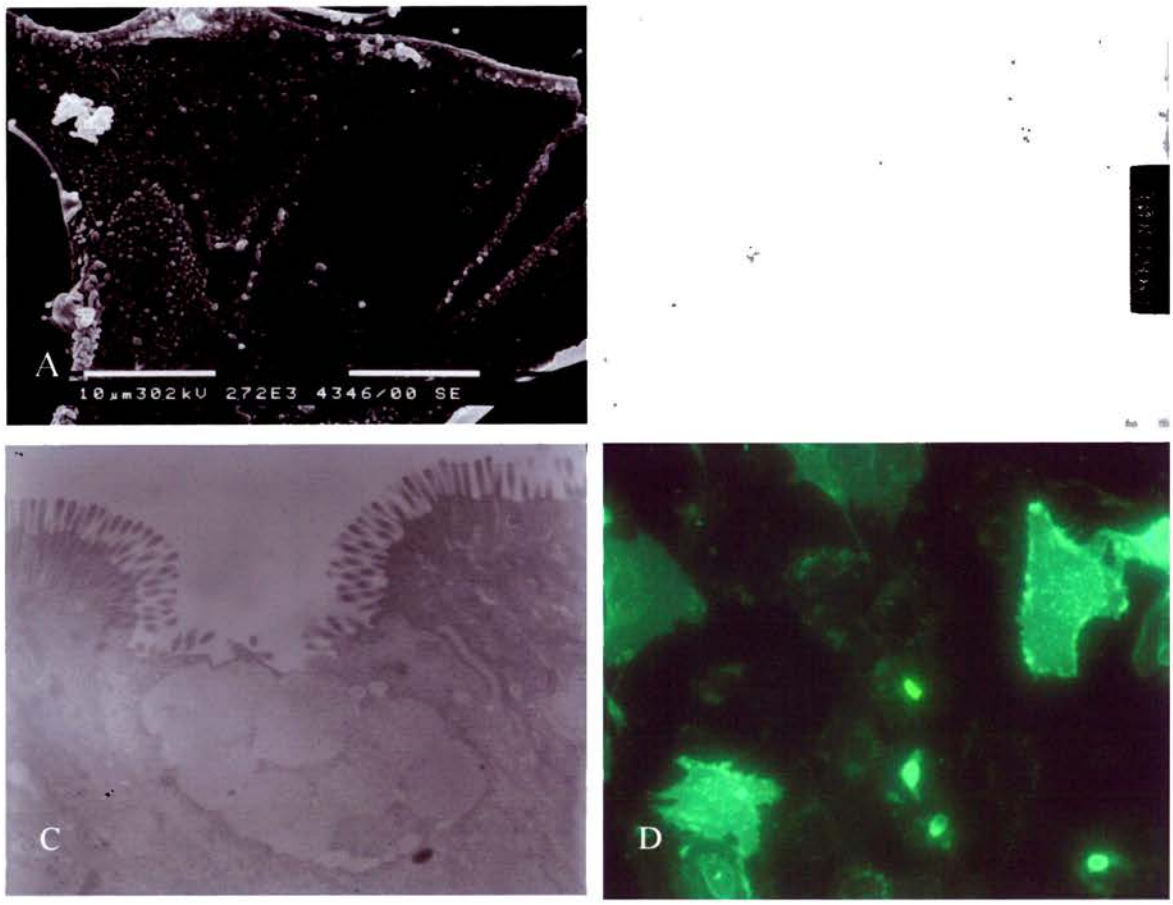

Fig. 3.3 Ultrastructure and phenotyping of primary cell culture. (A) Scanning electron micrograph showing uniform dense microvilli on the apical surface of primary epithelial cells. (B) Transmission electron micrographs through epithelial monolayer demonstrates presence of tight junctions (arrow) between the adjacent cells (x 13 600). The cells are polarized with microvilli on the apical surface. (C) Note the presence of a goblet cell in the monolayer (x 8700). (D) Immunofluorescence picture showing expression of Gb3- positive cells in the monolayer, marked as green punctate structures on the apical surface of few cells. The cells labelled with FITC-conjugated VT1 B-subunit were fixed with 2% PFA and examined using Leica DMLB epifluorescence microscope (x200).

Mucosal scrapings from the lymphoid-dense terminal rectal mucosa yielded a heterogenous population of crypts. In culture a minority of the crypts generated columns of cells that mainly expressed the intermediate filament protein vimentin (Fig. 3.4A, B). Additionally some crypts in the culture yielded only a few vimentin-expressing cells (Fig. 3.4C, D) although most crypts did not stain for vimentin. The number of crypts yielding vimentin-positive cells varied from 4-6% amongst replicates. On scanning electron microscopy some of the cells in the epithelial monolayer had short, sparse and stunted microvilli in comparsion to the surrounding cells which had more compact, regular and long microvilli typical of absorptive intestinal epithelial cells (Fig. 3.5). In the primary cell cultures, from crypts isolated from mucosa 20 to 25 cm proximal to the recto-anal junction, vimentin- expressing cells were not observed in the epithelial monolayer.

Uptake of inert microparticles has been used as a functional assay for M-cells in in vitro cultures (Kerneis *et al.*, 1997) as well as in vivo studies. To assess if the cells in primary culture were capable of taking up beads, this assay was done with latex fluorescent particles. After 45 minutes of incubation, fluorescent beads were seen adhering to a small proportion of the cells in the monolayer (Fig. 3.6A). On confocal laser scanning microscopy of the samples, microparticles were observed on the surface of cells as well as either being endocytosed or inside the cell (Fig. 3.6B). Co-localization studies revealed that the cells binding to microparticles also expressed intermediate filament protein vimentin (Fig. 3.6C, D). These cells capable of uptaking microparticles were observed in culture until 10-14 days. Not all vimentin-positive cells bound the beads however, out of all the cells present, only the ones stained with vimentin were found to bind the beads. The numbers of vimentin-expressing cells binding the beads were variable in different cultures and their number was maximum on 5 to 8 th day post-culture when about 10 % of these cells bound beads.

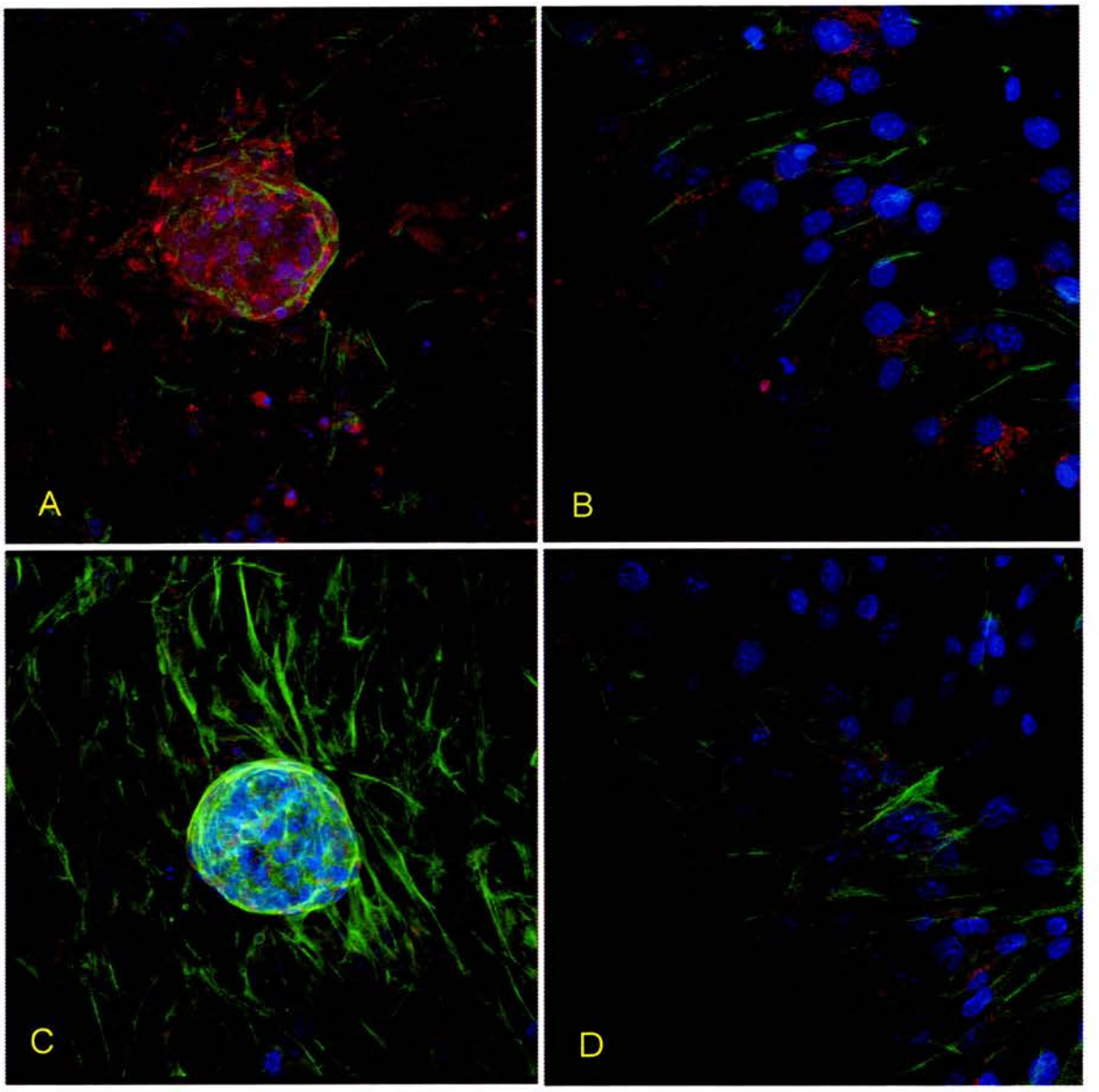

Fig. 3.4 Heterogeneous population of crypts in the primary cell culture from bovine terminal rectum. A minority of isolated crypts in culture were characterised by the presence of a subset of vimentin-positive cells. Five day old cultures were labelled with anti-vimentin (red), actin specific phalloidin-FITC (green) and nuclear stain TO-PRO (blue). (A-B) A mixed population of cells generated from the crypt. The majority of cells emerging from the crypt expressed vimentin. (C-D) A crypt with only a few cells expressing vimentin. The pictures were acquired using a Leica TCS NT confocal system (x63 objective).

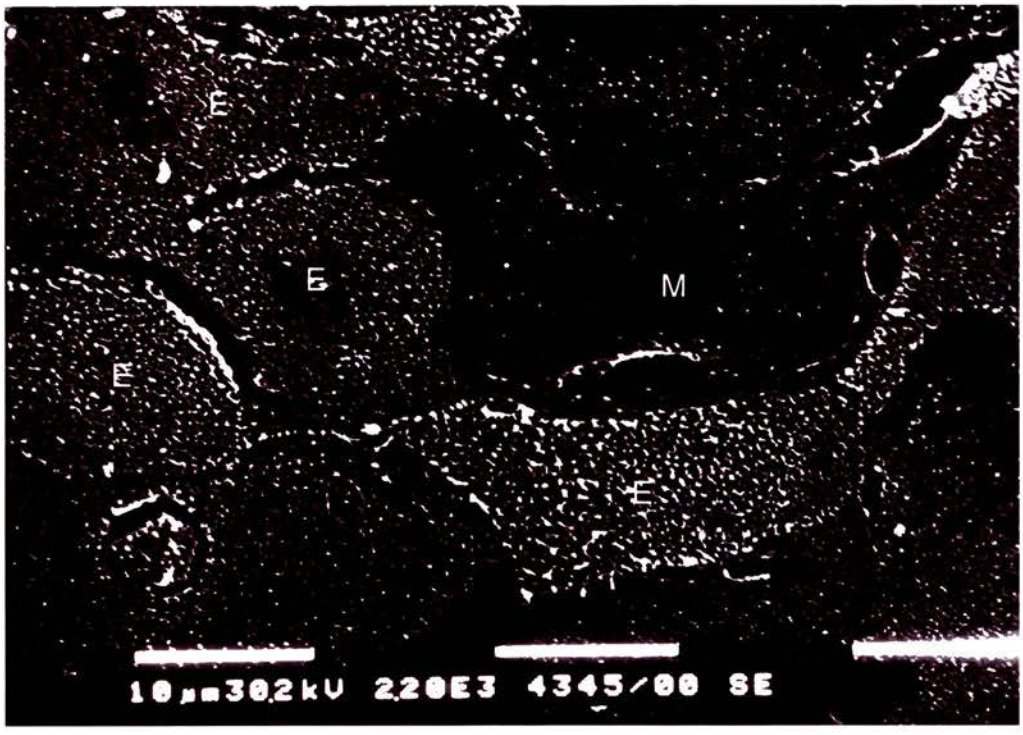

Fig. 3.5 Scanning electron micrograph of primary cell culture (6 day culture). The majority of cells demonstrated long, regular and compact microvilli on their apical surface, typical of absorptive enterocytes (E) which surrounded a single cell with short and sparse microvilli on the apical surface, apparently resembling M-like cell (M).

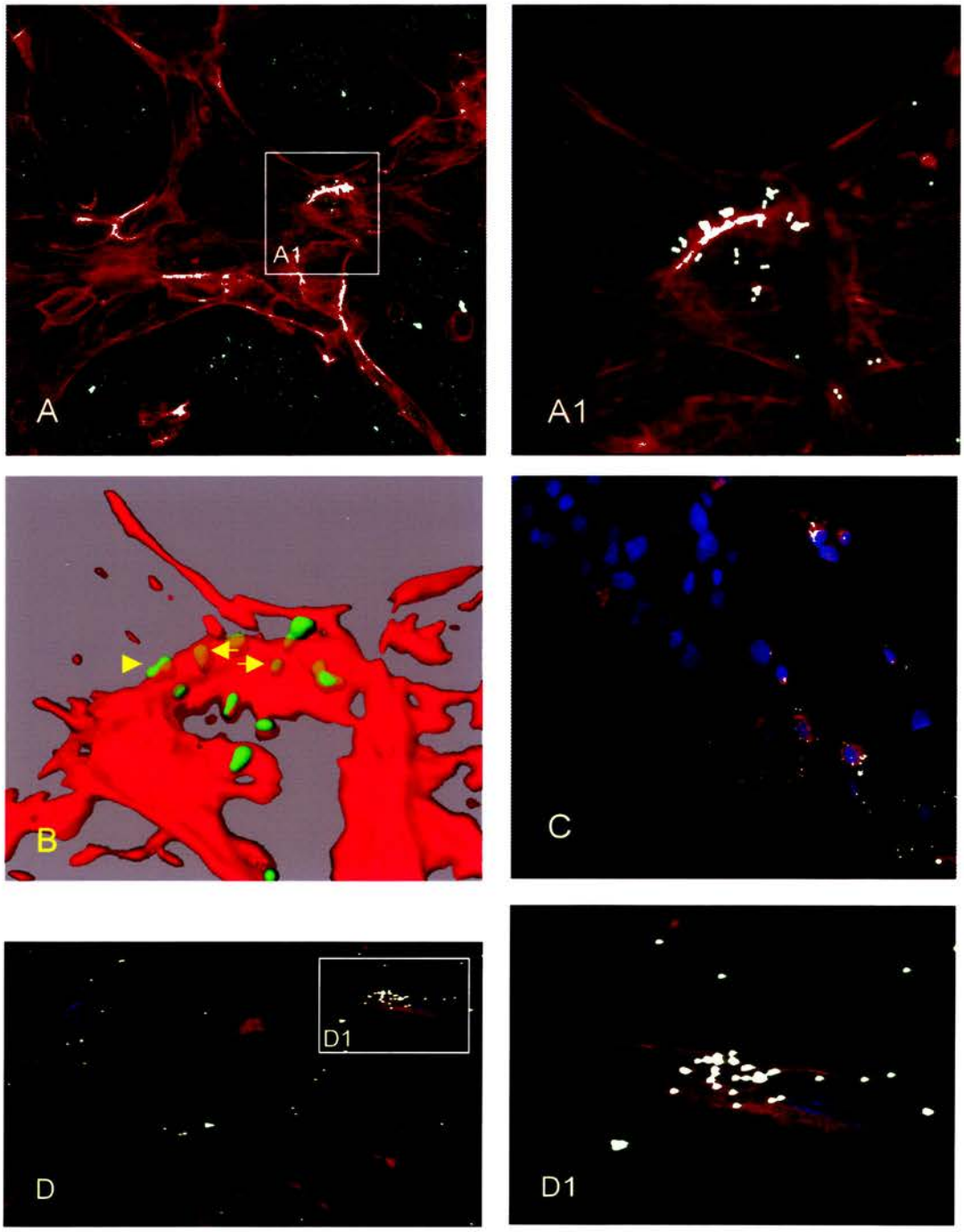

Fig. 3.6 Microparticle uptake assay and colocalization with vimentin. The primary rectal epithelial cells (7 day old culture) were incubated with latex particles (green) fixed, permeabilized and labelled with phalloidin-TRITC (red) in Fig 6 A, B or anti-vimentin (red) and TO-PRO (blue) in Fig C, D. The images were sequentially acquired, as serial sections of 0.1 μm thickness, using a a Leica TCS NT confocal system (x63). Image (A) and the digitally magnified inset image (A1) shows latex particles adhered to a cell in the monolayer. (B) A three-dimensional digital image of this cell, convoluted using a computer software programme Imaris Surpuss Module (Bitplane) demonstrated the internalization of microspheres. A few microparticles were completely internalized (arrow) and others were being internalized by the cell (arrow head). (C-D) A minority of the cells expressing vimentin (red) adhered to the fluorescent microparticle. (D1) The inset image shows the clustering of microparticles on a vimentin positive cell.

The non-quantitative immunofluorescence studies revealed that the bacteria (ZAP198-GFP) adhered to epithelial cells in culture with no particular preference to those expressing vimentin (Fig. 3.7). These bacteria adhered diffusely to many epithelial cells within the culture. To assess if the cells in the primary culture, capable of taking up beads, could bind to bacteria, the adherence assay was followed by colocalisation with microparticles. Although the presence of bacteria (ZAP198-RFP) and beads colocalised on some of the cells (Fig. 3.8) the bacteria adhered to many other cells. Further investigations are required to define the significance of this observation.

To confirm if MALT cells are present in the primary culture that might induce crypt cells to commit to FAE enterocytes or M-cell like phenotype, the epithelial monolayer was stained for various immune cell markers (Table 3.1). However, no staining was observed for any of the eight markers tested on cells at 72 h of culture.

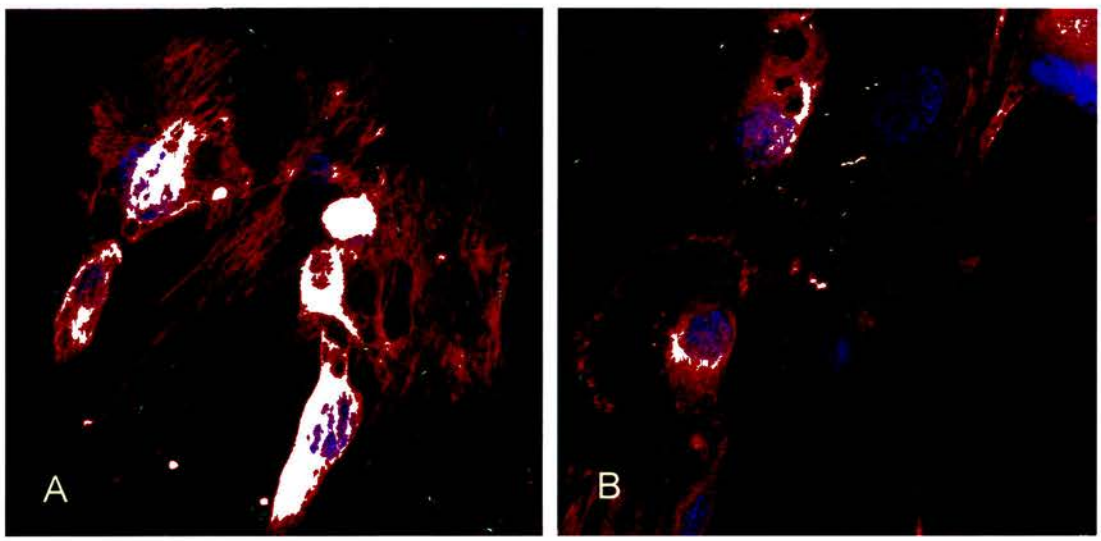

Fig. 3.7 Adherence assay with *E. coli* O157:H7 and colocalization with vimentin. The primary rectal epithelial cells were infected with *E. coli* O157:H7 (ZAP 198-GFP) at MOI 1:100 with bacterial cultures in mid-log phase of growth at 37°C, 5% CO_2 for 1 hr. The cells fixed/permeabilized (4%PFA/ 0.1% Triton X-100) were stained with anti-vimentin monoclonal antibody followed by TRITC- labelled secondary (red). The cellular actin was stained with phalloidin-Alexa 647 (blue) and visualised by using a Leica TCS NT confocal system (x63). Bacteria diffusely adhered to epithelial cells expressing or deficient in vimentin.

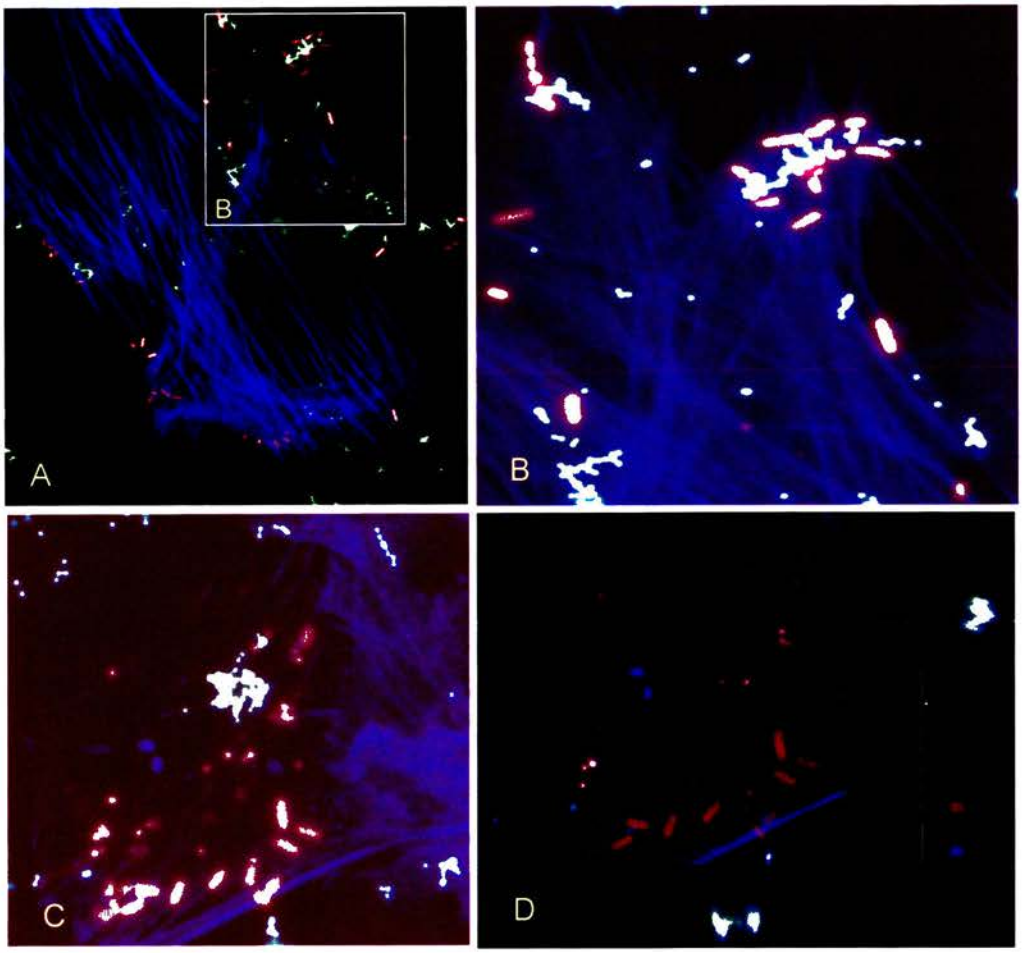

Fig. 3.8 Adherence assay with *E. coli* O157:H7 and microparticle uptake assay. The primary rectal epithelial cells were infected with *E. coli* O157:H7 (ZAP 198-RFP) at MOI 1:100 with bacterial cultures in mid-log phase of growth at 37°C, 5% CO_2 for 1 hr. The infected cells were further incubated with latex particles (green) for 45 min, fixed, permeabilized and labelled with phalloidin-Alexa 647 (blue) and visualised by using a Leica TCS NT confocal system (x63). The fluorescent beads (green) colocalised with adherent bacteria (red) on some of the cells (A-C). The image in Fig. B and C is digitally magnified by a factor of 6. Fig. C acquired as optical sections of 0.5 μm thickness, was viewed in the xz-plane that shows the location of beads on the cell surface (D).

3.4 Discussion

Epithelial cells of mucosal surfaces are comprised of multiple cell types, with distinct functions. For instance, intestinal epithelium contains absorptive, Goblet, entero-endocrine, Paneth and M-cell types. These cell lineages arise from pluripotent stem cells present in the crypts. Crypts contain clonal units of stem cells which undergo a limited number of divisions, which migrate bi-directionally and mature into epithelial cell types. The immature first-to-third-generation daughter cells of the stem cells are capable of stem cell function if necessary. Each crypt contains approximately 250 cells depending on the species and the anatomical location.

Of the epithelial lineages, the origin of M-cells is most contentious, as various studies support their derivation from enterocytes via extrinsic stimuli (lumenal antigens and/or lymphoid-follicle derived signals) or origination from specialised stem cells. To gain a clearer understanding of M-cell ontogeny and function, reliable, convenient and reproducible methods are required for isolating and culturing these cells in vitro.

A number of enzymatic procedures have been used for isolation of crypts in primary cell culture (Flint, Cove, and Evans, 1991). The present technique used a collagenase-dispase combination adapted from (Booth *et al.*, 1995) and (Hoey *et al.*, 2003b), which is a relatively gentle technique that gives a high yield of crypts, preserves the cell and crypt integrity and releases the epithelial units from underlying connective tissue with very few stromal cells attached. Any lamina propria cells released as single cells were removed on differential centrifugation in 2% sorbitol. The use of low level of serum (Hoey *et al.*, 2003b) and MEM-D valine culture media eliminated the contaminating fibroblasts (Hoey *et al.*, 2003b;Frauli and Ludwig, 1987;Lazzaro *et al.*, 1992). The isolated intact crypts adhered well to the collagen-coated surface and proliferated to produce sustainable cultures. After 4 days in culture, both flattened, thin, migratory cells and a compact, higher density, cobblestone monolayer could be seen around the crypts still attached to the collagen matrix. Mainly cells within the crypt, and a few of those in the developing monolayer, were proliferating as demonstrated by PCNA staining. These cultures

resembled native epithelium, i.e. proliferating crypt cells giving rise to non-proliferating enterocytes. As time progressed, crypt bodies became fewer as cells proliferated and migrated, eventually forming a monolayer. The cells in the monolayer expressed cytokeratins, formed tight junctions and were polarized with microvilli on the apical surface.

Epithelial lineages differ in a range of phenotypic characteristics including the type of intermediate filament proteins and surface glycoconjugates expressed (Gebert and Posselt, 1997;Giannasca *et al.*, 1999;Clark *et al.*, 1993;Gebert and Hach, 1993b). For example intermediate filament protein vimentin, cytokeratins 8 and 18, are found in rabbit, rat and pig intestinal M-cells, respectively (Jepson *et al.*, 1992;Gebert, Hach, and Bartels, 1992;Rautenberg *et al.*, 1996). M-cells, in the terminal rectum of cattle, have been demonstrated to express vimentin as the predominant intermediate filament protein (Mahajan A *et al.*, 2005). Apical surface glycoconjugates have also been frequently used as markers for M-cells but there is no clear-cut common marker for these cells irrespective of the species and the location in the gut. For example in mice, UEA-1 is a marker for M-cells in Peyer's patches but not in the colon (Valdivia and Falkow, 1996).

In primary culture there was a mixed population of crypts. A small but reasonably consistent proportion of crypts in primary cultures from lymphoid-follicle-dense rectal mucosa expressed vimentin as an intermediate filament protein although the number of crypts varied in different cultures. The vimentin-expressing crypts in culture as seen in Fig. 3.4A is reminiscent of crypts associated with follicle (referred to as follicle-associated crypts) as observed on immunohistological studies on bovine terminal rectal tissue (Mahajan A *et al.*, 2005). This shows that crypts which generated a large population of vimentin-expressing cells in culture are derived from the follicle-associated crypts, which stained positively for vimentin in situ. On isolation in culture, these crypts generated a cohort of vimentin-expressing cells which may be deduced to be precursors of FAE enterocytes or M-cells, stained strongly for vimentin in the lymphoid-dense terminal rectal mucosa of cattle (Mahajan A *et al.*, 2005). The follicle- associated crypts in GALT of rabbits have

been shown to express vimentin-positive cells as precursors of M-cells. These cells emerge in columns and migrate to apical surface of adjoining follicles to constitute the FAE and M-cells (Lelouard *et al.*, 2001b).

M-cells are distinctive epithelial cells that are structurally and functionally specialized for transepithelial transport, delivering antigens and microorganisms from the lumen to the organised lymphoid tissue within the mucosae. This functional characteristic of M-cells has been examined in various in vivo and in vitro studies (Kerneis *et al.*, 1997;Pappo and Ermak, 1989;Landsverk, 1988;Porta *et al.*, 1992a). In this primary culture of rectal epithelial cells, a subset of vimentin-expressing cells endocytosed latex fluorescent microparticles in an *in vitro* functional assay for M-cells in culture (Kerneis *et al.*, 1997). On the basis of these findings it may be inferred that the cells expressing vimentin in culture are the population at various stages of committment of differentiation into M-cells.

Epithelial cells emerging from crypts, isolated from the rectal mucosa 18-20 cm proximal to anorectal junction, neither expressed vimentin as an intermediate filament protein nor trancytosed beads when tested comparatively. In this region of the bovine rectum, organised lymphoid follicles were not observed (Mahajan A *et al.*, 2005). Therefore, it appears that only the follicle-associated crypts from a lymphoid dense mucosa on isolation in culture yielded cells with the characterstics of M-cells.

There is a mounting body of evidence that MALT cells or the factors produced by them regulate the differentiation of FAE and M-cells both in in vitro lympho-epithelium co-culture system (Kerneis *et al.*, 1997)and in vivo (Kerneis *et al.*, 1997;Goosney, DeVinney, and Finlay, 2001a;Sharma *et al.*, 1996). In the present system of primary cell culture from crypts, isolated from lymphoid-dense mucosa, few of the cells differentiated to a M-cell-like phenotype. It is a matter of further investigation whether in the culture the crypt stem cells were pre-commited or were previously induced by the surrounding lymphoid microenviornment in situ, to differentiate into M-cells. Such pre-commited or preconditioned/preinduced crypts

on isolation in culture might have yielded cells functionally and phenotypically similar to M-cells. Although in this culture system mucosal-associated lymphoid cells were absent when tested post 72 h of culture; the presence of other soluble factors like lymphokines, which have been documented to influence M-cell differentiation even in absence of lymphocytes (Debard *et al.*, 2001), cannot be ruled out. It can be proposed that if the lymphoid cells are regulating the M-cell differentiation, this event is happening at very early stages of crypt stem cell differentiation.

Previously, attempts have been made to culture M-cells in vitro using either tissue explants of lymphoid rich mucosal tissue or isolating these cells on enzymatic digestion of lympho-epithelium (Pappo, Steger, and Owen, 1988;Roy and Ruiz, 1986). But since M-cells are end-stage differentiated cells that cannot divide (Bye, Allan, and Trier, 1984), these in vitro models could only be used briefly. Most of the FAE/M-cell related in vitro studies involve a range of cell lines established from a number of common human adenocarcinomas. Although these cells have many advantages, they still have major constraints in both being derived from abnormal tissue and being subjected to repeated selection pressure of tissue culture. Furthermore, these cells, in the absence of other cell types and a complex extracellular matrix normally associated with the epithelium, are in a compromised environment far from the normal situation. This work may offer a novel method for in vitro culture of M-cell-like cells in primary cell culture.

Since *E. coli* O157:H7 colonises lymphoid follicle-dense terminal rectum of cattle, the possible role of M-cells/FAE, the distinctive attributes of this region, is an interesting possibility that might drive this unique tropism. The presence of M-cells/ FAE-like cells in the primary culture provides a very relevent in vitro model to further these studies in identifying their role in *E. coli* O157:H7 colonization in the terminal rectum of the bovine host. In this study, the bacteria adhered to epithelial cells with no apparent preference to vimentin-positive cells or the subset of these cells taking up microparticles. Although bacteria adhered to some cells colocalised with beads, quantitative studies are required to establish significance of this

observation. Thus, although *E. coli* O157:H7 shows tropism for terminal rectum, a region rich in FAE and M-cells or vimentin-positive cells, it shows more generalised affinity for enterocytes from this region, although specific determinants for this interaction remain to be defined.

4 Role of verotoxin in interaction of EHEC strains with bovine rectal epithelium

4.1 Introduction

Shiga toxin(s) (Stx; or verotoxin VTs) are the major recognised virulence factors of enterohaemorrhagic *E. coli* (EHEC), the sub-group of Shiga-toxigenic *E. coli* (STEC) which are important causes of food-borne zoonosis (Mainil, 1999;Paton and Paton, 1998). EHEC strains possess multiple pathogenicity determinants including LEE (locus of enterocyte effacement) pathogenicity island and virulence-associated plasmid(s) such as pO157 of *E. coli* O157 (Nataro and Kaper, 1998) both of which encode pathogenicity factors. The main characteristic virulence factors of EHEC, the Shiga toxin(s), are encoded on lysogenic bacteriophage(s). There are several recognised Stx subtypes (principally Stx1, Stx2 and Stx2c in EHEC) encoded on multiple stx-bacteriophages which may be carried by EHEC strains (Boerlin *et al.*, 1999a).

Stx are a family of exotoxins that consist of a single enzymatically active A-subunit and five identical B-subunits responsible for toxin binding to the glycolipid Gb3 (globotriaosylceramide) receptor (Lindberg *et al.*, 1987;Lingwood, 1999), on the target cell (Lacy and Stevens, 1998;Sandvig, 2001). After binding of Stx, the toxin-receptor complex is internalized (Sandvig and van Deurs, 2000) and undergoes retrograde transport via the Golgi complex to the endoplasmic reticulum (ER) and the nucleus (Sandvig *et al.*, 1989;Arab and Lingwood, 1998). The A-subunit is translocated to the cytoplasm, where it exerts N-glycosidase activity and cleaves off a single adenine residue from the 28S rRNA of the 60S ribosomal subunit, leading to irreversible inhibition of protein synthesis that is toxic to the cell (Endo *et al.*, 1988). Although toxin sensitivity of a given cell correlates mainly with the level of Gb3 expressed on its surface, it has been shown that modifying the length of Gb3 fatty acid chains or dissociation of Gb3 from lipid rafts can alter intracellular trafficking of Stx, leading to premature termination of retrograde transport in the Golgi (Lingwood, Khine, and Arab, 1998;Sandvig *et al.*, 1992;Sandvig *et al.*, 1994) or transport to lysosomal compartments respectively (Falguieres *et al.*, 2001;Hoey *et al.*, 2003a).

EHEC infections in man are associated with enteric and systemic disease syndromes ranging from diarrhoea, to more severe diseases consequences including haemorrhagic colitis (HC) and haemolytic uremic syndrome (Paton and Paton, 1998). As currently understood, ingestion leads to colonisation of follicle-associated epithelium overlying the Peyer's patches (Phillips *et al.*, 2000b) and other epithelial sites. This leads to expression of LEE-encoded factors resulting in attaching and effacing (A/E) lesions and other responses. Stx are expressed *in situ* (Ball *et al.*, 1996;Caprioli *et al.*, 1995;Mackenzie *et al.*, 1998;Park *et al.*, 1996;Ramotar *et al.*, 1995;Stapp *et al.*, 2000) and activities include induction of apoptosis in Gb3-expressing cells. Human intestinal epithelial cells lack Gb3 (Holgersson, Jovall, and Breimer, 1991;Kasai *et al.*, 1985;Lingwood, 1996) and are thus resistant to Stx cytotoxicity however, despite the absence of receptor, Stx is a key determinant in pathophysiology of EHEC infections. First, Stx can cross intestinal epithelium by a pericellular route (Acheson *et al.*, 1996;Hurley *et al.*, 1999;Philpott *et al.*, 1997) and localise to sub-epithelial tissues. Once it has crossed the epithelial barrier, Stx can target Gb3-expressing endothelium of the mucosal vasculature leading to local damage, for example, presenting as haemorrhagic colitis. Secondly, a crucial action of Stx in the induction of proinflammatory mediators by epithelia, which damage the intestinal mucosa directly by inflammation and indirectly by promoting recruitment of phagocytes (Thorpe *et al.*, 1999;Thorpe *et al.*, 2001;Yamasaki *et al.*, 1999). Thirdly, polymorphonuclear leukocytes attracted to the site of EHEC colonization sequester Stx leading to their delivery to other tissues and facilitating systemic toxicity (Hurley, Thorpe, and Acheson, 2001;Te Loo *et al.*, 2000b). Gb3 is expressed by endothelium, monocytes and macrophages (Foster and Tesh, 2002;Molostvov *et al.*, 2001;Ohmi *et al.*, 1998;Ramegowda and Tesh, 1996;Yoshida *et al.*, 1999) and engagement with Stx can lead to cyotoxicity and/or induce cellular signalling leading to changes in gene expression (for example cytokines and adherence molecules (Molostvov *et al.*, 2001;Ramegowda and Tesh, 1996;Morigi *et al.*, 2001;Bitzan *et al.*, 1998;Jacewicz *et al.*, 1999), amplification of inflammatory processes and further sensitization to Stx by upregulation of Gb3 expression (Molostvov *et al.*, 2001). Stx thus possesses multiple activities of an effector and a cytotoxic molecule.

In contrast to man, cattle and other reservoir hosts typically carry EHEC asymptomatically (Chapman *et al.*, 1993;Synge, 2000). Despite the prevalence of EHEC in cattle, a role of Stx in this host has yet to be ascribed although Stx can be detected in biologically-relevant levels in cattle faeces (Ball *et al.*, 1994;Hyatt, Galland, and Gillespie, 2001) and Gb3 is expressed by bovine intestinal epithelium both in situ and primary epithelial cell cultures (Hoey *et al.*, 2003a;Hoey *et al.*, 2002). However, Stx is not toxic to Gb3-positive bovine intestinal epithelial cells in primary epithelial cell culture (Hoey *et al.*, 2003a). Differential cytotoxicity might arise through structural variation in Gb3 (Falguieres *et al.*, 2001) which contribute to membrane localisation, transmembrane signalling and dictate intracellular trafficking of Stx either to endosomal/lysosomal compartments (where toxin is functionally neutralised) or to endoplasmic reticulum and nucleus (resulting in protein synthesis inhibition and apoptosis). In bovine intestinal epithelial cells although Stx localises, and is detoxified, in endosomal/lysosomal compartments (Hoey *et al.*, 2003a) it has also been documented to modulate intestinal inflammation (Menge *et al.*, 2004), thus possibly assisting the "commensal" life style of STEC in this reservoir host.

Intestinal epithelial cells (IEC) are at the interface of host-pathogen interaction and hence constitute the first line of defence. Bacterial interaction with IECs result in up-regulation of number of proinflammatory cytokines including IL-8 (Jung *et al.*, 1995;Yang *et al.*, 1997). IL-8, a member of CXC chemokine family, is a potent neutrophil chemoattractant which not only recruits neutrophils to the site of infection but also stimulates several responses in these cells essential for microbial killing. These include release of granule constituents such as proteases, reactive oxygen intermediate products and other proinflammatory products and thus neutrophils that migrate to gastro-intestinal tract in response to infection can subsequently damage the intestinal mucosa (Grisham and Granger, 1988;Wallace *et al.*, 1992). In the case of human STEC infections, elevated IL-8 levels have been shown to correlate with both leukocytosis and severity of disease (Fitzpatrick *et al.*, 1992;Murata *et al.*, 1998;Westerholt *et al.*, 2000). Moreover it has been demonstrated that Stx can induce a number of epithelial pro-inflammatory cytokines including IL-8 via a ribotoxic

stress pathway (Thorpe *et al.*, 2001). Therefore, recruitment of polymorphonuclear leukocytes (PMNs) subsequent to IL-8 induction is a central hallmark of STEC infections in human beings.

STEC strains are non-invasive pathogens, however the passage of Stx into underlying tissues is a crucial step in pathogenesis and PMN are now thought to be important in this process. Recently it was shown that, due to an increase in para-cellular permeability and break down of the tight junction barrier, the amount of Stx1 and Stx2 crossing polarized T84 monolayers (Gb3-negative cells) in an apical-to-basolateral direction was proportional to the magnitude of PMN migration (Hurley, Thorpe, and Acheson, 2001). PMN have been shown to bind Stx2 directly *in vitro* and *in vivo* via a receptor with a 100-fold lower affinity than Gb3. Bound toxin was subsequently released on contact with target cells expressing Gb3 (Te Loo *et al.*, 2000a). Thus, PMNs may function in the pathogenesis of STEC diseases by contributing to the breakdown of the intestinal epithelial barrier and as well by transporting Stx to target tissues. Hence much of the intestinal pathology associated with STEC diseases, as well as life threatening complications, results from microvascular angiopathy due to systemic absorption and dissemination of Stx.

Although a substantial amount of data have been generated in recent years regarding the interaction of STEC with epithelial cells using *in vivo* and *in vitro* models, so far there has been no study aiming specifically at the role of Stx in interaction with the gut epithelium of cattle, the primary reservoir host for these organisms. Since *E. coli* O157:H7 exhibits a novel tropism for the terminal rectum in the bovine host (Rice *et al.*, 2003b;Naylor *et al.*, 2003;Sheng *et al.*, 2004a) this work aimed to investigate the role of Stx in colonization of STEC strains using bovine primary rectal epithelial cells as an in vitro model. The influence of Stx on synthesis and secretion of IL-8, a key mediator in pathology of STEC infections in humans was also examined.

4.2 Materials and Methods

4.2.1 Bacterial strains

Serotypes of *E. coli* strains used in this study are summarised in Table 1. ZAP 196 (VT2+) and ZAP 198 (VT2-) are *E. coli* O157:H7 isogenic strains of human origin, that were isolated from the same patient in an outbreak in which cattle were identified as the source of infection (Ostroff *et al.*, 1990). ZAP 198 was naturally cured of VT2 bacteriophage. Strain ZAP 270 (VT1+VT2+) (serotype O157:H7), an isolate from Sakai outbreak in Japan (Fukushima *et al.*, 1999) and the non-toxigenic isogenic mutant ZAP 273 were a gift from C. Sasakawa, Japan. EHEC (O103:H2) strains ZAP 268 (VT1+), the isogenic VT mutant ZAP 269 (Jenkins *et al.*, 2003b) and the wild type *E. coli* O157:H7 strains (ZAP 3, ZAP 41, ZAP 58) were of bovine origin. Strains were genotyped for key EHEC determinants as described previously (McNally *et al.*, 2001). EPEC strain used in this study was ZAP 195 (O80).

Table 4.1. Genotype and serotype of EHEC strains used in this study

Reference No	Serotype	Ehx	espB	VT1	VT2	VT2c	VT2v
ZAP0196	O157:H7	+	+	-	+	-	+
ZAP0198	O157:H7	+	+	-	-	-	-
ZAP0268	O103:H2	+	+	+	-	-	+
ZAP0269	O103:H2	+	+	-	-	-	-
ZAP0270	O157:H7	+	+	+	+	-	-
ZAP0273	O157:H7	+	+	-	+	-	-
ZAP0003	O157:H7	+	+	-	+	+	+
ZAP0021	O26:H -	+	+	+	-	-	+
ZAP0041	O157:H7	+	+	-	+	+	+
ZAP0058	O157:H7	+	ND	-	+	ND	ND
ZAP0194	O26	+	-	+	-	-	+

ND: Not done

4.2.2 Primary cell culture

Bovine primary epithelial cells were cultured as described in Chapter 3 from the most terminal region of rectum. The cells were maintained in D-Valine medium (Gibco) supplemented with 1% or 2.5% batch-tested FCS, 0.25 U/ml insulin, 10 ng/ml EGF and 30 μg/ml gentamicin (Hoey *et al.*, 2003a). The cells were grown on collagen-coated 24-well culture plates or 4-well chamber slides (Costar, Corning USA) until

confluency with approximately $3x10^5$ cells/well. A state of confluency was reached approximately 10 to 14 days following initial primary epithelial cell culture.

4.2.3 Adherence assay

Bacterial colonies (3 to 4) on LB plates grown freshly from -70°C stock cultures were used to inoculate 5 ml of MEM-HEPES (Sigma). The cultures were incubated in a shaking incubator (37°C, 200 rpm) for about 18 h. These overnight bacterial cultures were diluted 1:10 in MEM-HEPES (Sigma) and grown to an optical density of 0.3-0.4 at OD_{600} in a shaking incubator (37°C, 200 rpm) for approximately 3 h. An aliquot (100 μl) of bacterial culture (approximately $3x10^8$ cfu/ml) was used to infect confluent bovine rectal primary epithelial cells in duplicate at a multiplicity of infection (MOI) 1:100 in MEM-HEPES. The primary epithelial cells prior to infection were washed two times and incubated in 400 μl of pre-warmed (37°C) MEM-HEPES medium. The cells were infected for 3 h at 37°C in a 5% CO_2 atmosphere. Non-adherent bacteria were removed by washing three times with phosphate buffered saline (PBS), and cells were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 30 min at room temperature. The infected cells were stained with DIFF-Quik staining kit (Dade, Behring Dudringen Switzerland) as per the manufacturer's instructions and the adherent bacteria were counted as described before (Boerlin *et al.*, 1999b). Briefly, a total of 100 cells were examined under the light microscope and the number of bacteria adherent to each cell were counted in randomly selected fields. More than twenty bacteria per cell were evaluated as a microcolony.

In some experiments to evaluate the effect of VT on adherence, the infections were done with the ZAP 198 (VT-ve) strain in presence of exogenous VT2 (100 ng/ml)(Sigma). The confluent primary epithelial cells (10 to 14 days post culture) were prepared as above. The monolayers were infected with ZAP 198 at a MOI 1:100 in presence or absence of VT2 in MEM-HEPES for 3 h at 37°C in a 5% CO_2 atmosphere. The non-adherent bacteria were removed by washing three times with phosphate buffered saline (PBS). The adherent bacteria were solubilised with PBS-0.1% (v/v) Triton X-100, serially twenty-fold diluted, and plated to estimate the number of bacteria adhering to the cell monolayers as colony forming units (cfu).

4.2.4 Statistical analysis

The data were analysed as Binomially distributed responses, where from each experimental replicate the number of cells with greater than 20 adhering bacteria was treated as the response variable. Data from 3 VT positive strains were analysed in this way, each paired with isogenic mutant strains. Hence, the strain-pair and VT status defined two explanatory factors. The procedure was carried out independently on three occasions, and the Series term defined this as a possible explanatory factor. These data were analysed using a series of Generalised Linear Models (McCullagh P and Nelder J.A, 1989), using a logit link function. There was no statistically significant evidence of over-dispersion in these data. The effect of including terms in the model was calculated using a chi-squared distribution to assess the statistical significance of the resulting changes in the model deviance.

4.2.5 FAS test

Actin rearrangement was detected by fluorescence actin staining (FAS) as described before (Knutton *et al.*, 1989)). Briefly, bovine rectal primary epithelial cells were grown to confluence on collagen-coated chamber slides. The epithelial cells were prepared and infected with different *E. coli* strains (ZAP 196, ZAP 198) at a MOI of 1:100 for 3 h at which time media was replaced with fresh MEM-HEPES and cells were incubated for a further 3 h at 37°C in 5% CO_2. The infected cells were then washed three times with PBS, fixed/permeabilized in 2% (v/v) Formalin-0.2% (v/v) Triton X-100 for 20 min at RT. The fixed cells were washed twice in PBS, adherent bacteria were stained with rabbit anti-O157 polyclonal antisera (1:500, Mast Diagnostics) at RT for 30 minutes and detected with FITC-labelled secondary antibody (1:1000, Sigma). Cells were then treated for 30 minutes at RT with TRITC-Phalloidin (5 μg/ml; Sigma), washed twice with PBS and mounted in fluorescent mounting medium (DAKO). The specimens were visualised under Leica DMLB epifluoresence microscope.

4.2.6 Electron microscopy

Bovine rectal primary epithelial cells were grown to a confluent stage on collagen-coated 13 mm Thermanox coverslips (Nalge Nunc International, Rochester USA). The confluent epithelial cells were pre-washed and infected with ZAP 196 at MOI of 1:100 in MEM-HEPES, for 3 h at 37°C in 5% CO_2 as described above. The infected

cells were washed three times with PBS to remove the non-adherent bacteria. The cells were fixed with 3% (v/v) gluteraldehyde in 0.1% (v/v) sodium cacodylate buffer at pH 7.4 for 2 h at 4°C and processed for scanning and transmission electron microscopy as discussed in Chapter 3.

4.2.7 Preparation of bacterial supernatants

Freshly grown bacterial colonies from -70°C stock cultures were used to inoculate 5 ml of MEM-HEPES (Sigma). The cultures were incubated overnight in a shaking incubator (37°C, 200 rpm). These overnight bacterial cultures were diluted 1:50 in MEM-HEPES and grown to an optical density of 0.6-0.7 at OD_{600} in a shaking incubator (37°C, 200 rpm). The bacterial cultures were centrifuged at 4000 rpm for 15 min and the resultant supernatants were filtered sterilized (0.22 μm) and aliquots stored at -20°C.

4.2.8 Challenge of bovine rectal primary epithelial cells with bacterial strains and supernatants

The bovine rectal primary epithelial cells were grown on collagen-coated 24-well tissue culture plates to a stage of confluence. Prior to challenge, the epithelial cells were cultured in D-Valine medium (Gibco) for 24 h in the absence of serum and other supplements. The epithelial monolayers were prepared and infected with different bacterial strains as summarised above (Table 4.1). The infected cells were washed three times in PBS to remove the non-adherent bacteria. The cells were lysed in 1 ml of RNA lysis buffer (Qiagen Sciences, Maryland USA) and stored at -20°C.

For challenge with bacterial supernatants the primary epithelial cells were prepared as discussed above and challenged with bacterial supernatants diluted 1:5 in MEM-HEPES for 3 h at 37°C in 5% CO_2. The epithelial cells were prepared and lysed in RNA lysis buffer as discussed above. The assays were done in triplicates and repeated at least on three occasions.

4.2.9 RNA isolation, determination of IL-8 mRNA by RT-PCR

RNA was extracted from bovine primary terminal rectal epithelial cells using RNeasy (Qiagen Inc) according to the manufacturer's instructions. An aliquot (0.2 μg) of extracted total RNA was reverse transcribed in a total reaction volume of 20 μl

according to the manufacturer's instructions (Reverse Transcription kit, Promega, Madison, Wisconsin). All PCR reactions were carried out in 50µl reaction volumes containing 5µl of cDNA, 200µmol of deoxynucleotide mix (dATP, dTTP, dCTP and dGTP), 25pmol of each primer, 5µl PCR buffer A (Promega), 1.5M $MgCl_2$, and 1 U Taq (Promega)using thermal cycler (Hybaid). Primer sequences used were based on previously published sequences: IL-8 (sense, 5'-TTCACAGCACTCGGAATCCT-3'; antisense, 5'-ATGACTTCCAAACTGGCTGTT-3') (Boudjellab *et al.*, 1998), and γ-actin (sense, 5'-ACCAACTGGGACGACATGGA-3'); antisense, 5'GAGCTTCTCCTTGATGTCAC -3') (Aldwell, Wedlock, and Buddle, 1996). The amplification reaction was carried out for a total of 35 cycles as follows: 94°C for 45 s, 56°C for 1 min and 72°C for 1 min, with a precycle of 94°C for 4 min and final extension at 72°C for 10 minutes. Each experiment included negative controls in which cDNA was omitted from the PCR reaction. 2 µl of the resulting PCR products were separated by electrophoresis in a 1.2% agarose gel stained with ethidium bromide.

4.2.10 IL-8 ELISA

IL-8 assays were performed on culture supernatants from confluent primary epithelial cells challenged with bacterial supernatants from panel of VT-expressing and their isogenic VT- mutant EHEC strains. Prior to challenge with bacterial supernatants, cells were incubated for 24 h in D-valine medium (Gibco) in the absence of serum and other supplements. Bacterial supernatants prepared in MEM-HEPES were diluted 1:2 with D-valine and the cells challenged for 12 h (250 µl per well). Bovine IL-8 concentrations were determined by the Quantikine IL-8 sandwich ELISA (R & D System, Abington, UK). This ELISA kit is intended for detection of human IL-8 however the manufacturer confirmed cross-reactivity with bovine IL-8 and the system has been used successfully by Boudjellab *et al.*, (Boudjellab *et al.*, 1998). Standard dilutions (0-2000 pg/ml of recombinant human IL-8 was used to determine concentrations of bovine IL-8 equivalent in cell culture supernatants. Results were presented as means±standard deviations (SD). Differences between samples were analysed for significance using the unpaired "Student's t-test" ($p \leq 0.05$).

4.3 Results

4.3.1 Effect of VT on adherence of EHEC strains to bovine rectal primary epithelial cells

The binding of EHEC strains to continuous epithelial cell lines has been extensively studied and well characterized. EHEC strains produce a characteristic histopathology in vitro and in vivo, termed the attaching and effacing (A/E) lesion characterized by disassembly of microvilli in infected cells and the formation of actin-rich pedestals underneath adherent bacteria (Frankel *et al.*, 1998).

In this study EHEC strains of human and bovine origin were used to examine the interaction with the primary epithelial cells from terminal rectum, the principal site of *E. coli* O157:H7 colonization in cattle. Confluent primary epithelial cells after 10-14 days of culture were infected with different bacterial strains grown to a mid-exponential phase at a MOI of 1:100 for 6 h at 37°C, 5% CO_2 and the adherence characteristics were examined using electron or immunofluorescence microscopy.

In Fig. 4.1 different stages during interaction of ZAP 196 with the epithelial cells could be seen. Electron micrographs depicted bacteria making an initial contact with the microvilli on the apical surface of epithelia cell(s) (Fig. 4.1A, C) followed by formation of a compact microcolony (Fig. 4.1B), and the disassembly of the microvilli (Fig. 4.1D). Characteristic A/E lesions with the bacteria intimately attached to the pedestal-like structures identified as intense spots of actin were visible by fluorescence actin staining (FAS) (Fig. 4.2).

To examine whether the presence of VT affects the formation of A/E lesions, adherence was performed with *E. coli* O157:H7 strains ZAP 196 (VT2+) and the isogenic non-toxigenic strain ZAP 198. The formation of A/E lesion was visualized by FAS. At 6 h post-infection both the strains ZAP 196 (VT2+) (Fig. 4.2A, B) and toxin mutant strain ZAP 198 (Fig. 4.2C, D) formed typical A/E lesions characterised by actin pedestals though the latter were sparse.

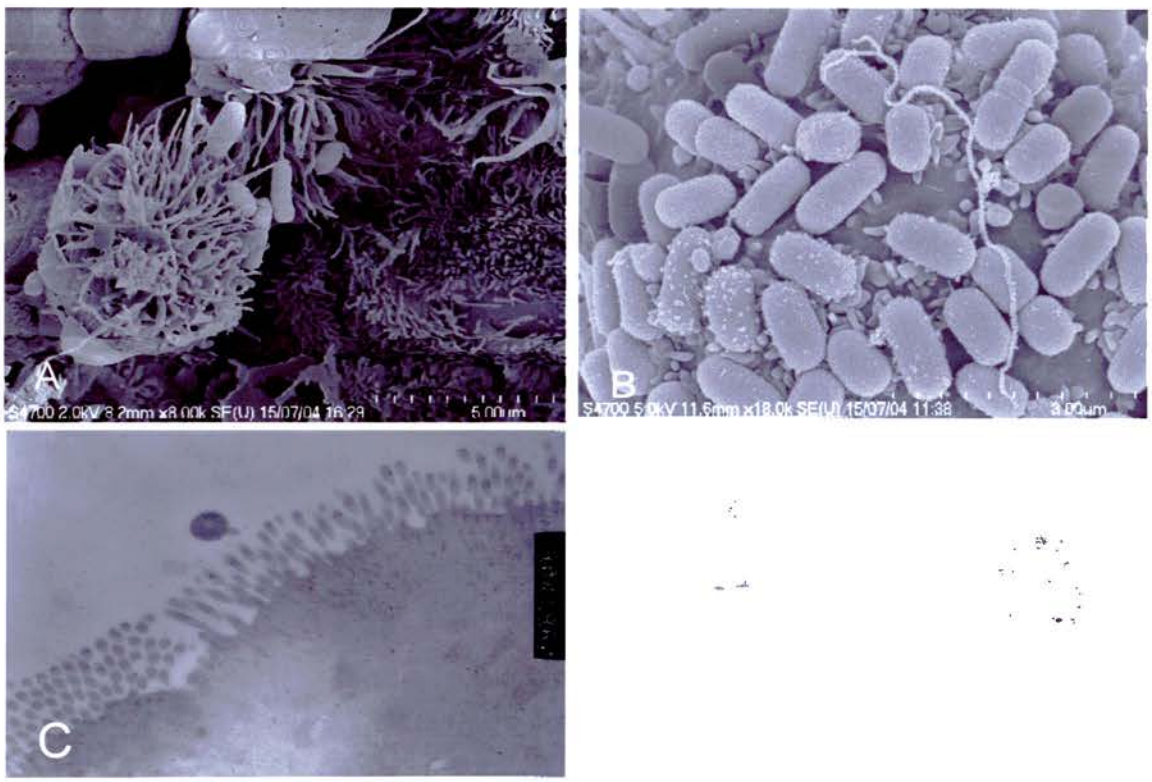

Fig. 4.1 Adherence of *E. coli* O157:H7 (ZAP 196) to the bovine rectal primary epithelial cells. Cells grown on collagen coated thermonax coverslips were infected with bacterial culture in mid-log phase of growth at 37°C, 5% CO_2 at a MOI 1:100 for 3 h. The infected cells were fixed with 3% (v/v) gluteraldehyde and processed for electron microscopy. Scanning electron micrographs depicting bacteria interacting with microvilli on the apical surface of cells (A) and formation of a compact microcolony (B). Transmission electron micrographs a of bacterium making an initial contact with the microvilli (x10500) (C) and causing its disassembly, the apparent beginning of attaching and effacing lesion formation (x 37200) (D).

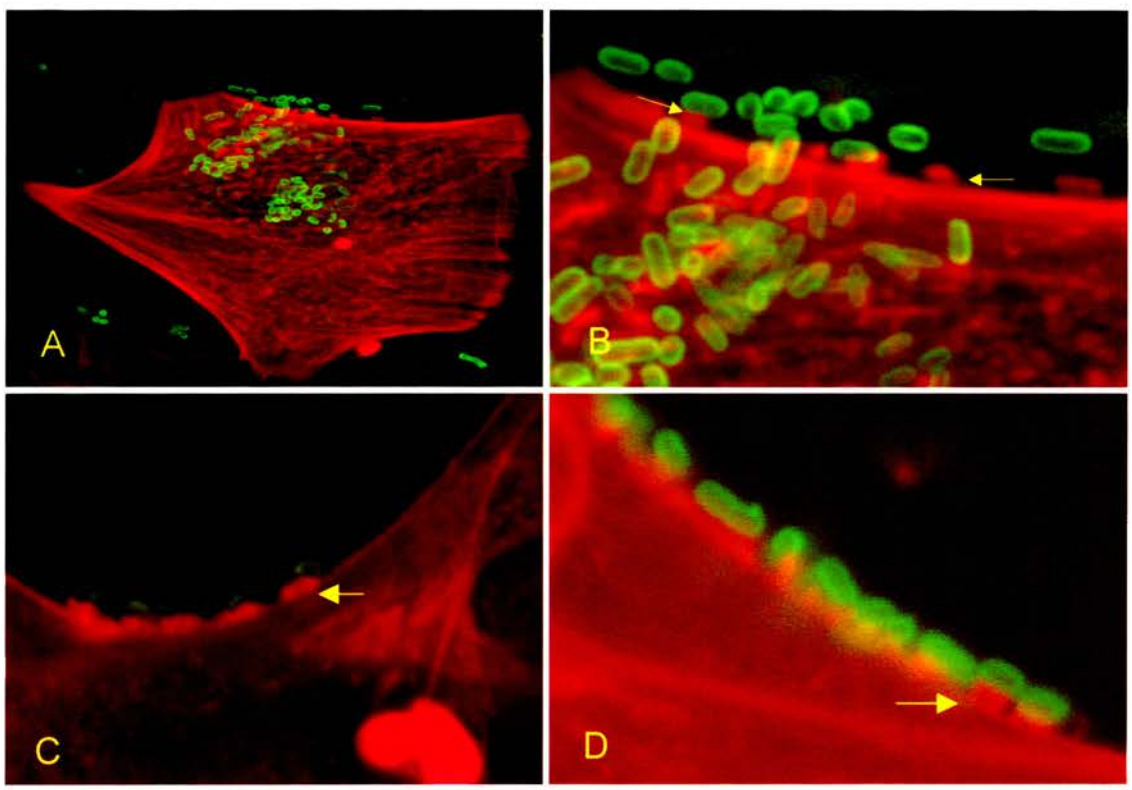

Fig. 4.2 Immunofluorescent micrographs showing characteristic attaching and effacing (A/E) lesions. The bovine rectal primary epithelial cells were infected with wild type *E. coli* O157:H7 strains ZAP 196 (VT2 +) (A-B) and the isogenic non-toxigenic strain ZAP 198 (C-D) for 6 hrs. The infected cells were fixed/permeabilized in 2% (w/v) PFA/ 0.2% (v/v) Triton X-100. The bacteria were stained with rabbit anti-O157 antisera and detected with FITC-labelled secondary antibody. Cells were stained with actin-specific TRITC-Phalloidin and examined using a Leica TCS NT confocal microscope (x63 objective). Images in Fig. (B, C, D) have been digitally magnified by a factor of 6 using Zeiss 510 confocal image software. Rearrangement of actin at sites of adherent bacteria (arrow) could be seen in form of raised pedestals or condensed actin spots.

To examine further the effect of VT on adherence of EHEC strains, a panel of VT-positive and their isogenic mutant strains (Table 4.1) were used in adherence assays on bovine rectal primary epithelial cells. Confluent primary epithelial cells post 10-14 days of culture were infected with these bacterial strains grown to a mid-exponential phase at a MOI of 1:100 at 37°C in 5% CO_2 for 3 h. The infected cells stained with Diff-quick stain were examined under light microscopy and the bacteria adherent to cells counted. At 3 h post infection the VT-expressing strains and their isogenic toxin mutants exhibited localized or diffuse adherence respectively as depicted with the representative strains ZAP 196 vs ZAP 198 (Fig. 4.3), ZAP 196 (VT2+) formed compact microcolonies characteristic of localized adherence (Fig. 4.3A), and ZAP 198 adhered sparsely resembling the diffuse adherent phenotype (Fig. 4.3B). The statistical analysis of the numbers of adherent microcolonies from the total sample of cells examined revealed that although the mean proportion of micro-colonies was subject to substantial variability between different experiments ($p \leq 0.001$), VT expressing strains ZAP 196, ZAP 268, ZAP 270 formed significantly higher mean proportions of microcolonies relative to their respective VT isogenic mutants (Fig. 4.3C) ($p \leq 0.001$). Note that the size of this effect was statistically significantly different for different strains ($p \leq 0.001$), the mean differential in the ZAP196/198 pair being smaller than that seen in the other strains. Nevertheless, these results indicated that overall, carriage of VT bacteriophage was associated with higher microcolony formation.

The effect of VT on absolute adherence was examined. For this the adhesion assay was done with a ZAP 198 (VT-deficient strain) with or without concomitant exogenous VT2 (100 ng/ml) for 3 h. The average cfu per ml for adherent ZAP 198 was $5.53 \times 10^6 \pm 2.14 \times 10^6$ (S.D) which was significantly increased to $1.86 \times 10^7 \pm 1.29 \times 10^7$ (S.D) ($p \leq 0.0001$) with addition of VT2 (Fig. 4.4).

4.3.2 EHEC infection induced IL-8 mRNA expression in bovine primary terminal rectal epithelial cells

To examine the role of EHEC organisms on expression of IL-8 by bovine gut epithelium, primary epithelial cells from terminal rectum of cattle were challenged

with different EHEC serotypes and the IL-8 expression was examined using RT-PCR. The epithelial cell cultures were infected with *E. coli* O157:H7 strains ZAP 3, 58, and 41; *E. coli* O26 strains ZAP 21 and 194 and EPEC ZAP 195 at MOI of 1:100 for 3 h and total cellular RNA was extracted as described in Materials and Methods. Equal amounts of total RNA from each sample were subjected to RT-PCR analysis with specific bovine IL-8 and γ-actin primers, respectively. Fig. 4.5 showed that all EHEC and EPEC strains induced IL-8 mRNA expression in the terminal rectum epithelium. The uninfected control cells did not show expression of IL-8.

4.3.3 Role of verotoxin in IL-8 mRNA expression at the bovine terminal rectal epithelium

To determine the role of verotoxin (VT) in EHEC induced expression of IL-8, the terminal rectal epithelial cells were challenged with the panel of VT-producing and their isogenic toxin mutant strains (Table 4.1). Primary cells were infected with *E. coli* O157:H7 strains ZAP 196 (VT2+) and isogenic VT- negative, ZAP 198; *E. coli* O103 strains ZAP 268 (VT1+) and its isogenic VT-negative ZAP 269. All strains induced expression of IL-8 transcript, however toxin-negative EHEC strains

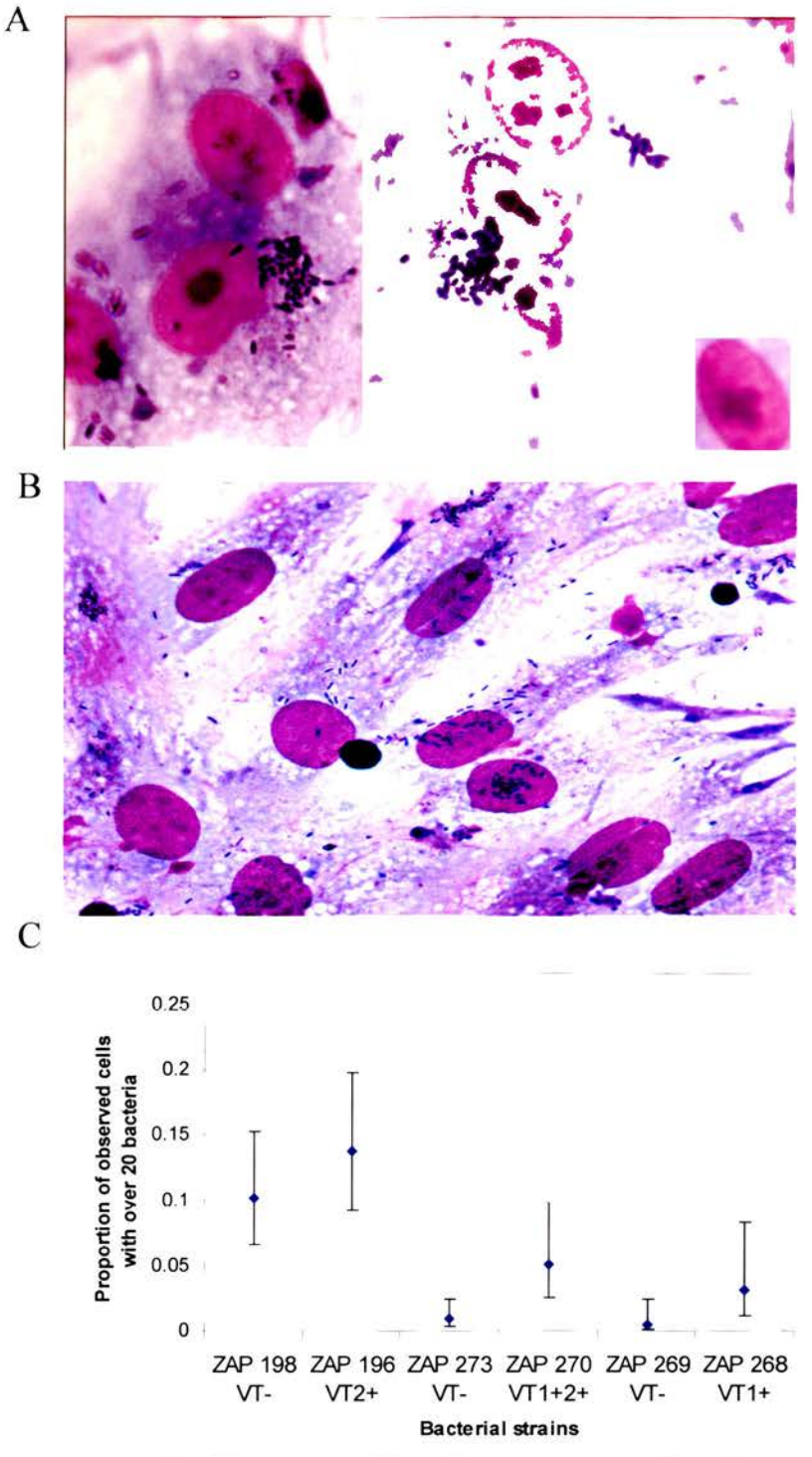

Fig. 4.3 Adhesion assay with verotoxin (VT) expressing and their isogenic mutant EHEC strains. The primary epithelial cells were infected at MOI 1:100 with wild type verotoxigenic and their isogenic toxin mutant strains at 37°C, 5% CO_2 for 3 hrs. The cells were stained with Diff-Quik stain and level of adherence quantified microscopically. The (A) and (B) are representative of adherence pattern by VT+ and VT- EHEC strains. ZAP 196 (VT2 +) formed compact microcolonies (A) and the isogenic mutant ZAP 198 (VT2-) were dispersed over the surface of the cell (B). (C) This graph depicts the effect of carriage of VT on formation of microcolonies. The ability to produce verotoxin by EHEC strains is associated with higher formation of microcolonies. This effect is consistent across all three strain pairs studied in the experiment ($p \leq 0.001$). The images were acquired using Leica DMLB epifluorescent microscope with a 100x (A) and a 20x (B) objective.

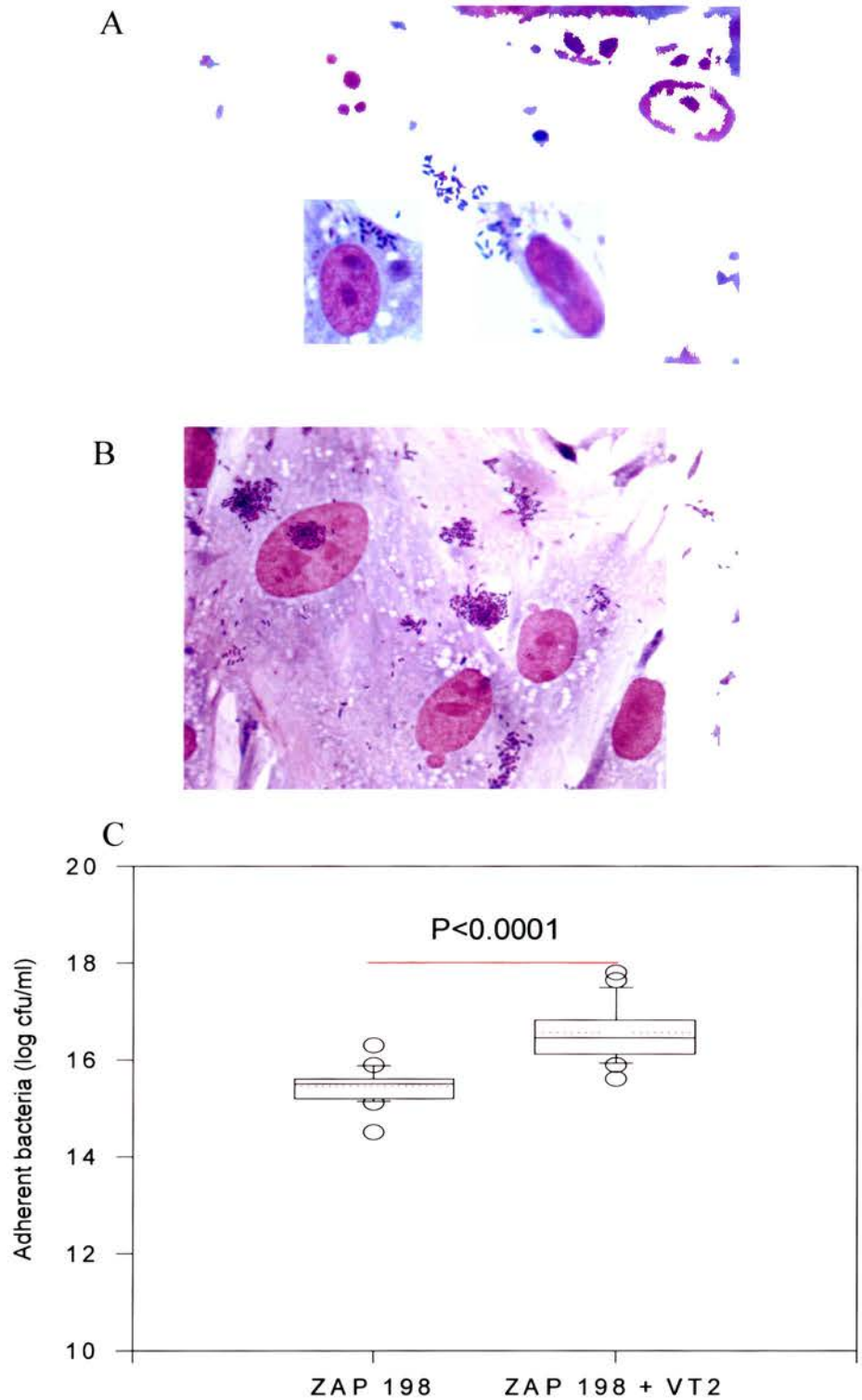

Fig. 4.4 Effect of verotoxin (VT) on adherence of VT-deficient *E. coli* O157:H7 (ZAP 198) strain. The bovine rectal primary epithelial cells were infected at MOI 1:100 with ZAP 198 in presence or absence of VT2 (100 ng/ml) at 37°C, 5% CO_2 for 3 hrs. The non-adherent bacteria were removed on washing with PBS. The adherent bacteria were removed with PBS-0.1% Triton X-100, serially diluted and enumerated as colony forming units (cfu). Box plot shows that VT significantly enhanced absolute adherence of ZAP 198 (C). Unpaired student's t-test was used to compare the groups statistically (n=2). Box plots: the solid line within the box is the median and the dotted line is the mean; the top and the bottom solid lines of the box represent the 75th and 25th percentiles, respectively, and the small lines outside the top and the bottom of the box represent the 90th and the 10th percentiles, respectively. Diff-Quik stained micrographs show pattern of adherence. The images were acquired using Leica DMLB epifluorescent microscope with a 100x objective. ZAP 198 formed microcolonies (A) which markedly increased in presence of VT (B).

appeared to induce a greater IL-8 mRNA expression after 3 h infection compared to their isogenic VT-positive counterparts (n=3 experiments) (Fig. 4.6A).

Challenge of the primary cells with bacterial supernatants from the same EHEC strains for the same time showed an identical pattern in IL-8 mRNA expression (n=3 experiments) (Fig. 4.6B). However VT alone did not induce IL-8 expression. These data imply that the secreted factors are the pro-inflammatory agonists and both VT1 and 2 down-regulate the expression of the chemokine IL-8 evoked by EHEC or its components/secreted factors.

4.3.4 Role of VT on secretion of IL-8 by bovine rectal primary epithelial cells

To quantify further the effect of VT on synthesis of IL-8, primary epithelial cells were challenged with bacterial supernatants from VT-expressing and their isogenic VT-mutant EHEC strains. The IL-8 protein levels were analysed using ELISA. The supernatants from all the EHEC strains induced variable levels of IL-8 which were significantly higher by VT-deficient strains in comparison to their wild-type VT-expressing strains: ZAP 196 (170.5 pg/ml) vs ZAP 198 (205.3 pg/ml); ZAP 268 (146.2 pg/ml) vs ZAP 269 (187.9 pg/ml) and ZAP 270 (187.8 pg/ml) vs ZAP 273 (343 pg/ml) (Fig. 4.7A).

To further evaluate the role of VT in suppression of IL-8, the bacterial supernatant from ZAP 198 was spiked with varying concentrations of VT1 ranging from 50-500 ng/ml and the IL-8 protein levels were measured. At these concentrations VT1 itself did not increase IL-8 secretion above control levels, indicating that VT1 is not itself recognised as pro-inflammatory by these cells. ZAP 198 supernatant produced IL-8 (237 pg/ml) which was not significantly different when spiked with 50ng/ml of VT (181 pg/ml). However at both 200 and 500ng/ml, VT1 suppressed IL-8 secretion (70 pg/ml and 0.001 pg/ml respectively) elicited by ZAP 198 supernatants to control levels, revealing a strong dose-dependent inhibitory effect of VT on IL-8 secretion at the terminal rectal epithelium in cattle (Fig. 4.7B).

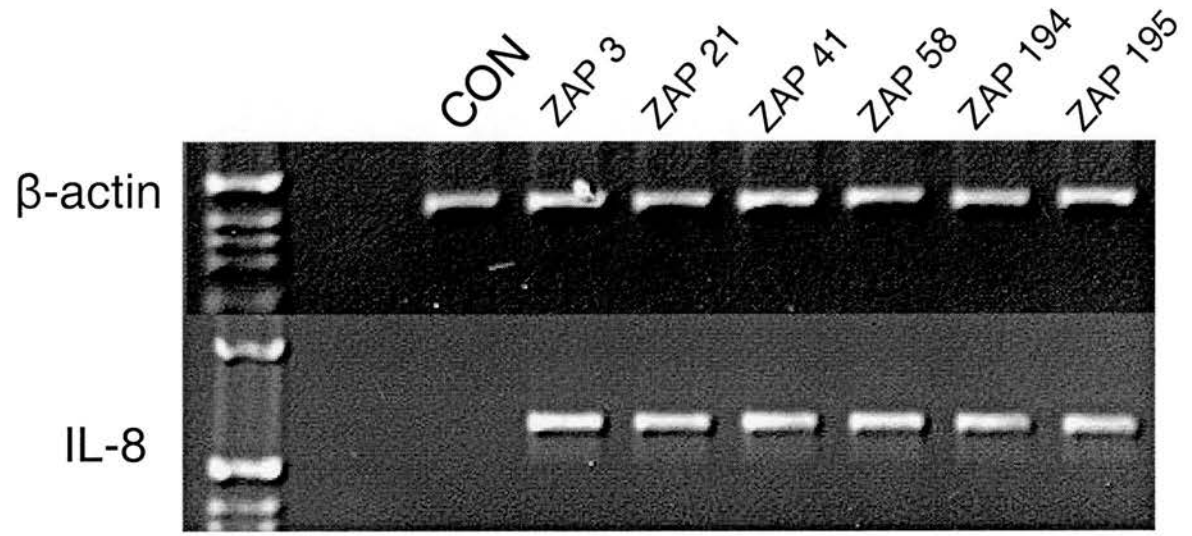

Fig. 4.5 VTEC and EPEC infection up-regulates IL-8 mRNA expression by primary cultures of bovine terminal rectal epithelial cells. Shown is an ethidium-bromide stained 1.2% agarose gel following RT-PCR analysis of total cellular RNA extracted from primary epithelial infected with EHEC serotype *E. coli* O157:H7 (ZAP 3, ZAP 58, ZAP 41), *E. coli* O26:H- (ZAP 21, ZAP 194) and EPEC isolate ZAP 195 (O80); each at a MOI of 1:100 for 3hrs. Non-infected cells were used as a control. Bovine IL-8 and β-actin primers generated fragments of 643 and 420 bp, respectively. The images were acquired using Alpha Innotech Imaging system.

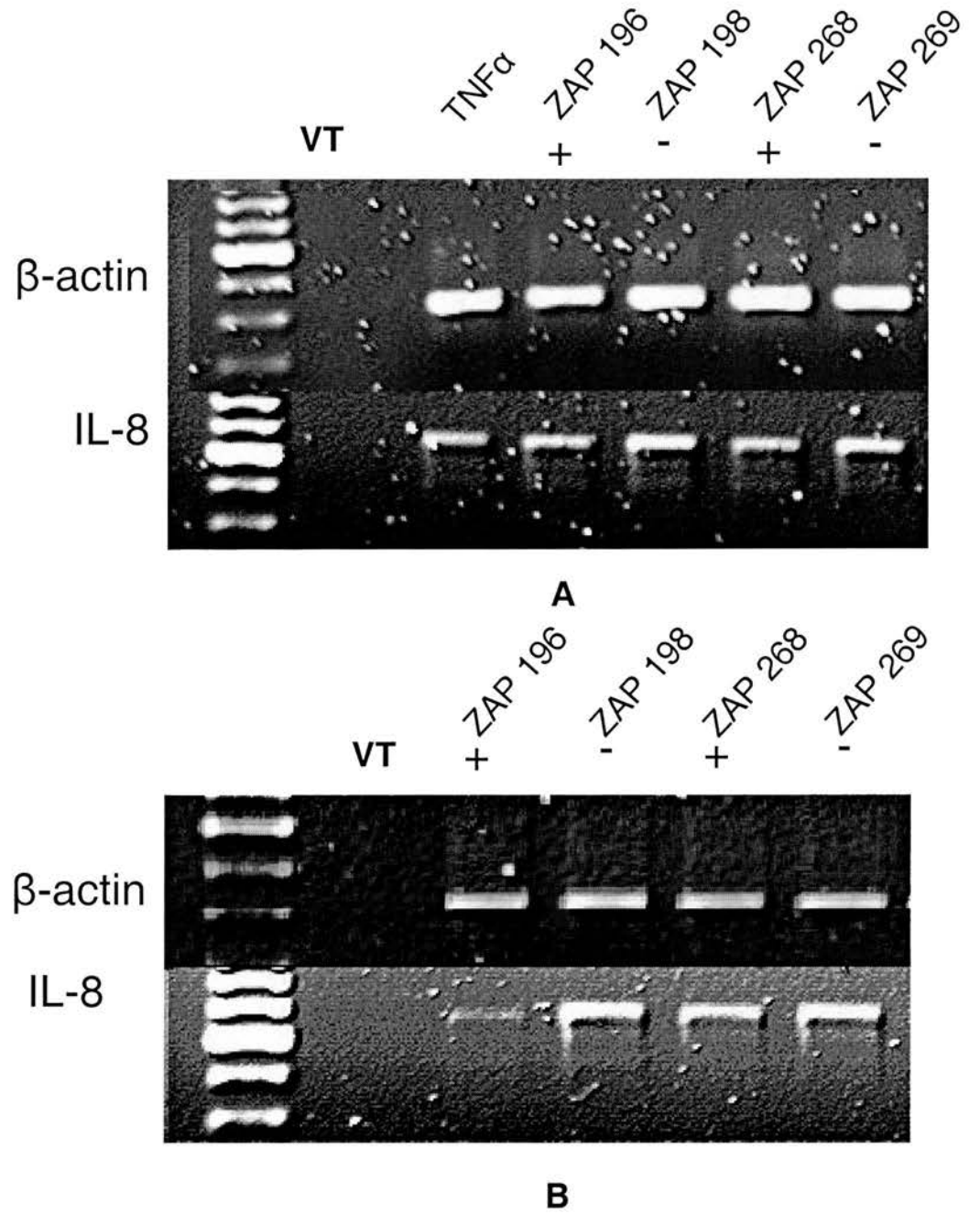

Fig. 4.6 Role of verotoxin (VT) in EHEC induced up-regulation of IL-8 mRNA in primary cultures of bovine terminal rectal epithelial cells. Shown is an ethidium-bromide stained 1.2% agarose gel following RT-PCR analysis of total cellular RNA extracted from primary epithelial cells infected with vero-toxigenic and their isogenic toxin mutant EHEC strain. (A) *E. coli* O157:H7 strains ZAP 196 (VT2+) vs ZAP 198 (VT2-) and *E. coli* O1O3:H2 strains ZAP 268 (VT1+) vs ZAP 269 (VT1-) or (B) with their respective supernatants. TNF-α (50ng/ml) was used as a positive control. Bovine IL-8 and β-actin primers generated fragments of 643 and 420 bp, respectively. RT-PCR analysis of mRNA expression showed that both toxin-negative EHEC strains and their respective supernatants induced a greater IL-8 mRNA expression after 3 hrs infection compared to their isogenic VT-positive counterparts. The images were acquired using Alpha Innotech Imaging system. (n = 3 experiments)

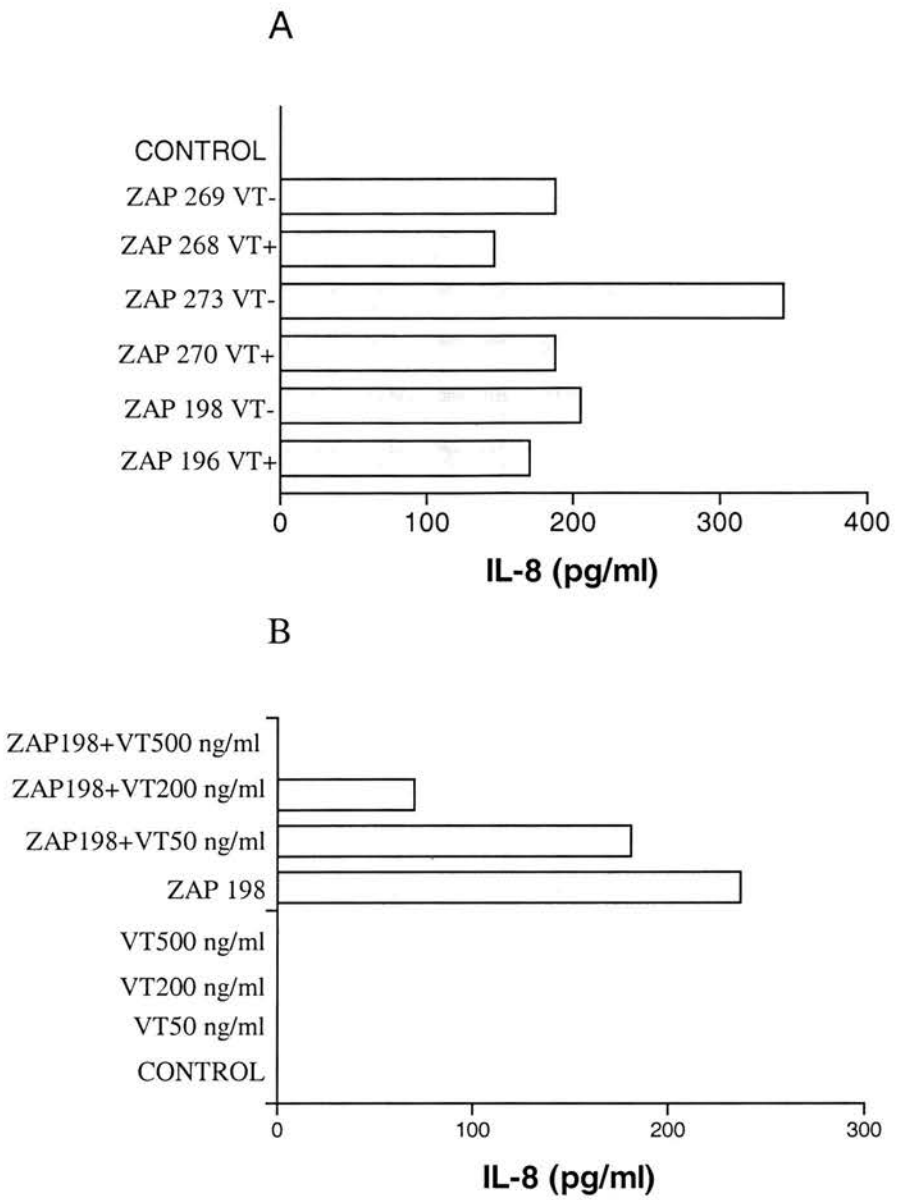

Fig. 4.7 Secretion of IL-8 by bovine rectal primary epithelial cells. The cells were challenged with (A) bacterial supernatants from vero-toxigenic (VT) and their isogenic toxin mutant EHEC strains: *E. coli* O157:H7 strains ZAP 196 (VT2+) vs ZAP 198 (VT2-) and ZAP 270 (VT1+ VT2+) vs ZAP 273 (VT-); *E. coli* O1O3:H2 strains ZAP 268 (VT1+) vs ZAP 269 (VT1-) for 12 hrs and IL-8 secretion in cell culture supernatants was measured by ELISA. Values are mean of three repeated experiments (n = 12 wells). IL-8 secretion levels were significantly lower with supernatants from VT-expressing strains than their respective isogenic toxin mutants.(B)) ZAP 198 bacterial supernatants were spiked with known concentrations of VT1 (50, 200 and 500ng/ml) and suppression of IL-8 secretion observed at 50, 200 and 500ng/ml (n = 4 wells). At these concentrations VT1 itself did not show any change in IL-8 secretion than the control.

4.4 Discussion

EHEC serotype *E. coli* O157:H7, an enteric human pathogen, has been incriminated in many serious outbreaks globally (Nataro and Kaper, 1998). The organism colonizes the intestine and produces multiple determinants which cause the pathology associated with the disease, with VT as a key factor in virulence. *E. coli* O157:H7 possesses a LEE pathogenicity island which is associated with the intimate adherence to epithelial cells, initiation of host signal transduction pathways, and formation of attaching and effacing intestinal lesions.

Intestinal colonization is a key aspect of infections caused by *E. coli* O157:H7 and many LEE and non-LEE encoded adherence factors have been documented to play a role in intestinal colonization of *E. coli* O157. LEE-encoded proteins including EspA translocon filaments, outer membrane protein intimin and the secreted protein-Tir are crucial to intimate adherence and colonization (Ebel *et al.*, 1998b) (DeVinney *et al.*, 1999). Other non-LEE encoded factors indicated to play role in *E. coli* O157:H7 adherence include ToxB and Efa 1 (Nicholls, Grant, and Robins-Browne, 2000;Stevens *et al.*, 2002a), LPS O-antigen side chains (Bilge *et al.*, 1996;Cockerill, III *et al.*, 1996), outer membrane proteins (Iha and OmpA) (Tarr *et al.*, 2000;Torres and Kaper, 2003) and fimbrial proteins (curli, LPF1, LPF2 and Loc 8) (Kim and Kim, 2004) (Jordan *et al.*, 2004;Dziva *et al.*, 2004). Hence *E. coli* O157:H7 adherence is complex and multifactorial. Several toxins e.g. Enterohemolysin EhxA, serine proteases EspP, metalloprotease StcE and EAST1 have been characterised in EHEC although verotoxins are essential characteristic determinants. Production of VT is an essential feature in many of the pathological conditions associated with EHEC infections in humans. Verotoxins act both as potent cytotoxins and as inducers of inflammation in the mucosa (HC) and systemically (HUS, TTP). An investigation of the association between virulence factors of STEC and disease in humans, indicated that the *stx2* gene is significantly associated with the severity of the disease (Boerlin *et al.*, 1999b). Although the toxic and proinflammatory activities of VTs in EHEC pathology have been investigated extensively *in vitro* and *in vivo*, the role of VT in colonization/adherence in particular has been an overlooked feature. Further

roles for VT in cattle have been largely disregarded despite the high incidence of STEC colonisation of cattle.

The terminal portion of the rectum has been identified as the primary site of colonization of *E. coli* O157:H7 in both naturally- and experimentally-infected cattle (Naylor *et al.*, 2003;Rice *et al.*, 2003a;Sheng *et al.*, 2004a;Stoffregen, Pohlenz, and Dean-Nystrom, 2004). Microcolony formation and attaching and effacing (A/E) lesions - features typically associated with initiation of inflammation - were noticeable at this site. Epithelium, in particular the terminal rectal epithelium, is thus an important site for interaction of EHEC with the bovine host. It has been shown previously that VTs are not directly cytotoxic to bovine intestinal epithelium (Hoey *et al.*, 2003a) therefore this study focused on the inflammatory responses at this site and how VT impacts on the relationship between EHEC and its bovine reservoir host. Consequently, primary cell cultures from the terminal rectal mucosa proximal to the recto-anal junction were used to assess the interaction of EHEC and VT with bovine intestinal epithelial cells *in vitro*.

First, this study aimed to investigate whether VT has a role in adherence of EHEC strains to the bovine gut epithelium. A panel of VT-producing and their isogenic toxin mutant strains were used in adherence assays on primary epithelial cells from terminal rectum. All the three VT-positive strains exhibited higher micro-colony formation, formed compact microcolonies, resembling typical localized adherence. In agreement with this, the addition of exogenous VT2 to cells concomitant with non-toxic ZAP 198 significantly enhanced bacterial binding.

Reports of toxins promoting adherence are few. However, the role of toxin has been documented in the colonization of *Clostridium difficile*. *C. difficile* strain ATCC43598 ($ToxA^-B^+$) producing toxin colonized better than the non-toxic ATCC43593 ($ToxA^-B^-$) during infection studies in mice (Tasteyre *et al.*, 2001). Previously, in a similar study, it was indicated that *C. difficile* toxins could influence *in vivo* colonization. Toxin-producing virulent strains (B-1) colonized gastro-intestinal tract of hamsters significantly higher than the avirulent non-toxigenic strain

(M-1). The administration of toxin along with M-1 strain significantly enhanced its adherence while inactivated toxin had no effect (Borriello *et al.*, 1988). Therefore enhancement of adherence by toxins is not unique to VT and EHEC but reports of this phenomenon are rare.

The interaction of VT with host cells can cause activation of host cell signalling pathways either directly or by stimulating host activating factors, such as inflammatory cytokines. Such activations could alter the host cell surface, thereby providing the EHEC with alternate novel receptors or enhance expression of constitutive receptors. Many pathogens rely upon host response to infection to activate a target receptor e.g. the adherence and invasion of human umbilical vein endothelial cells by *Streptococcus pneumoniae* is markedly increased following thrombin or TNF-α stimulation (Cundell *et al.*, 1995). *Borrelia burgdorferi*, the causative agent of Lyme disease, binds to the integrin $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb-IIIa) on human platelets but only if the platelets are activated (Coburn, Leong, and Erban, 1993). The toxin might affect adherence in a number of other ways: it may expose masked adherence sites; cell binding domain of toxin may enhance adhesion to epithelia; VT may increase exposure of phosphatidylethanolamine which act as ligands to certain bacterial adhesins and thus enhance binding (Barnett Foster *et al.*, 2000).

There are few reports which are indicative of possible role of VT in colonization by VTEC. One of the most important factors influencing *E. coli* O157:H7 infection is the low infectious dose, estimated at between 20 and 700 organisms in one study (Tuttle *et al.*, 1999) and less than 100 organisms in another study (Willshaw *et al.*, 1994). This is in marked contrast to EPEC which required an inoculum of $>10^8$ organisms to produce experimental infections in adults (Levine *et al.*, 1978;Donnenberg *et al.*, 1993). In another investigation, STEC serotypes isolated from a large food-borne outbreak were characterised for virulence-associated genes and compared in their ability to adhere to intestinal epithelial (Henle 407) cells. STEC strains from HUS cases showed enhanced adherence compared to the ones isolated from contaminated food source. In addition, the adherence of an O111 VT-

negative isolate was 72.2% compared with a 100% of VT-expressing O111-H STEC strains (Paton *et al.*, 1997).

In another study planned to determine the ability of *E. coli* O157:H7 to colonize and persist in sheep, animals were simultaneously inoculated with cocktail of three strains representing three pathotypes of *E. coli*. It was observed that *E. coli* O157:H7 strains persisted longer than ETEC and EPEC (Cornick *et al.*, 2000). Similar experiments done in pigs, showed that STEC strains expressing either *stx1* and *stx2* or only *stx2* were shed at significantly greater numbers and persisted over a longer period of time in comparison to EPEC and ETEC in co-infection studies (Booher, Cornick, and Moon, 2002). These studies inferred that toxin-producing VTEC may create a niche to facilitate persistence, in competition over other inoculated strains.

The intestinal tract is inhabited by a large number of diverse gut microflora and the interactions of these colonizing microbes with the host are complex. Once VTEC is introduced, can compete the resident gut microbiota and establish as a "commensal" in ruminant hosts (Cornick, Booher, and Moon, 2002b;Jordan *et al.*, 2004;Dean-Nystrom *et al.*, 1997;Naylor *et al.*, 2003). The local application of VTEC *E. coli* O157:H7 administered as rectal swab successfully colonized the rectal mucosa of cattle during the ruminant infection model studies (Sheng *et al.*, 2004a). The distinction of VT, along with other factors, may confer *E. coli* O157:H7 the advantage to compete over indigenous microflora and colonize successfully. *E. coli* O157:H7 has been documented to persist in cattle for as long as 6 months (Cray, Jr. and Moon, 1995). Experimental challenges of cattle with isogenic VT-positive and VT-negative *E. coli* O157 strains demonstrated a potential contribution of VTs *in vivo* through a trend towards enhanced colonisation by VT-positive strains (Naylor *et al.*, 2003), therefore from the current work it was hypothesised is that VTs contribute to colonisation and persistence possibly by local immunomodulation.

Verotoxins (VTs), major virulence factors of EHEC, play a key role in the patho-physiology of EHEC-induced infections in humans including bloody diarrhoea, haemorrhagic colitis (HC) and potentially fatal haemlolytic uremic syndrome (HUS).

Human intestinal epithelium lacks Gb3 (Holgersson, Jovall, and Breimer, 1991), but despite the absence of its receptor, VT is an important aetiological factor in mediating release of pro-inflammatory mediators from epithelial cells (Thorpe *et al.*, 2001).

Epithelial proinflammatory cytokines attract leukocytes that disrupt epithelial integrity as well as sequester VT and assist its systemic delivery (Hurley, Thorpe, and Acheson, 2001;Te Loo *et al.*, 2000b). In VTEC-infected humans, elevated levels of IL-8 has been incriminated in the observed leukocytosis and subsequent disruption of the intestinal epithelial cell barrier. Thorpe *et al.* reported that VT induced IL-8 expression and secretion in a human intestinal epithelial cell line (Thorpe *et al.*, 1999). An important role for VT could be the augmentation of the cytokine synthesis response to infection in the milieu of the sub-mucosa, from which EHEC strains are excluded. Cytokines synthesised in the sub-mucosal tissues may enhance recruitment and activation of inflammatory cells with subsequent compromise of the intestinal barrier, thereby further potentiating the systemic effects elicited by VTs.

In contrast to humans, cattle typically carry EHEC, particularly *E. coli* O157:H7, asymptomatically (Chapman *et al.*, 1993;Synge, 2000). Though VT has been detected at significant levels in faeces of cattle (Ball *et al.*, 1994;Hyatt, Galland, and Gillespie, 2001) and verotoxin-producing *E. coli* are prevalent in cattle (Boerlin *et al.*, 1999b) there is no evidence that VT is toxic to the adult ruminant host. One possible explanation for the lack of virulence in cattle may be that the Gb3-positive basal crypt cells in the intestinal epithelium sequester VT and processes it to endosomes/lysosomes where it is functionally neutralized (Hoey *et al.*, 2003a). But the frequent detection of anti-VT antibodies in the sera and colostrum of cows (Johnson, Cray, Jr., and Johnson, 1996;Pirro *et al.*, 1995) provides additional evidence that the persistence of bovine VTEC infections does not result from a general inability of cattle to respond to STEC or its products. VTEC could have evolved mechanism(s) that actively limit intestinal inflammation, maintain intestinal homeostasis, and finally allows persistent colonization. Therefore, it was

hypothesised that VTs are able to modulate intestinal inflammation in the bovine mucosa.

This work was planned to examine the effect of VT on IL-8 synthesis and secretion by bovine gut epithelium. Primary epithelial cells on challenge with a panel of wild type EHEC (including O157:H7 strains) and EPEC strains from both bovine and human origin, showed an increase in IL-8 mRNA expression relative to unchallenged cells. Pathogenic bacteria, including enteroaggregative *Escherichia coli* (Steiner *et al.*, 2000), EHEC (Dahan *et al.*, 2002), EPEC (Zhou *et al.*, 2003b), *Salmonella typhimurium* (McCormick *et al.*, 1993) and *Helicobacter pylori* (Crabtree *et al.*, 1994), to name a few, have long been associated with up-regulation of a number of pro-inflammatory cytokines and chemokines. IL-8 is one of the major factors secreted by epithelial cells as part of the acute innate response to infection, high levels of which have been shown to be indicators of the risk of HUS in EHEC-infected children (Westerholt *et al.*, 2000) and the observed leukocytosis and consequent pathology of the disease (Fitzpatrick *et al.*, 1992){Murata, 1998 984 /id . Different bacterial structural components or secreted proteins have been indicated to induce IL-8 expression but the relative importance of each remains to be elucidated and the literature is clearly divided on contribution of each of these factors. VT1 and VT2 have previously been shown to induce IL-8 production in human intestinal epithelial cells {Thorpe, 1999 437 /id}(Thorpe *et al.*, 2001;Yamasaki *et al.*, 1999). However, Berin *et al.* reported that the differences in responses elicited by the various VTEC strains in Hct-8 and CaCo2 cell lines were unrelated to the type or amount of VT produced and that VT was not required for a strong IL-8 response to infection with *E. coli* O157:H7(Berin *et al.*, 2002).

The role of other VTEC factors in IL-8 induction by bovine primary epithelial cells is currently under investigation. Other work from this lab has shown that, in contrast to human intestinal epithelial T84s, bovine primary intestinal epithelial cell cultures when challenged with VTs do not directly stimulate IL-8 gene expression or secretion from these cells. However, challenge of these cells for 3 h with VT-positive O157:H7 and O103:H2 strains or their respective supernatants demonstrated suppressed IL-8 expression and secretion relative to their isogenic VT-deficient

counterparts. In corroboration of this, supernatants from the O157:H7 VT-negative strain ZAP 198, when spiked with known concentrations of VT, showed a dose-dependent decrease in IL-8 secretion and a complete inhibition of IL-8 secretion at the highest dose used (500 ng/ml of toxin). However the biological significance of this finding still remains to be established in experimental calf studies. It is part of ongoing research to quantify the IL-8 levels and the type of immune cells at the site of *E. coli* O157:H7 colonisation in intestinal epithelium of calves challenged with verotoxigenic and isogenic VT-mutant strains.

These findings identify an apparent VT-dependent modulation of pro-inflammatory responses, a characteristic that was not predicted from recognised activities of this toxin or of EHEC. VT is not the only recognised bacterial factor reported to be involved in the suppression of cytokines. Yop B protein of *Yersinia enterocolitica* has been shown to inhibit secretion of TNF-γ from homogenates of Peyer's patches which aids its colonization in the mouse gut (Beuscher *et al.*, 1995). Supporting evidence showed that VTEC clinical isolates lacking *espB* significantly induced neutrophil migration across and IL-8 secretion from T84 intestinal cells relative to their *espB*-positive counterparts (Hurley, Thorpe, and Acheson, 2001). Recently Hauf *et al.* (Hauf and Chakraborty, 2003) reported that VTEC, EHEC and EPEC actively suppress bacterial- or TNFα- induced NF-κB DNA-binding activity in HeLa cells in an *espB*-dependent manner. Thus suppression of cytokines is a property common to many bacteria producing A/E lesions. Recently it was shown that Stx1 binds directly to intraepithelial lymphocytes and attenuates IL-8 synthesis in culture (Menge *et al.*, 2004). Thus, VT could influence the local immune homeostasis and in combination with other unknown factors facilitate the "commensal" life style, and explain prevalence of VTEC in cattle.

This work defines an immuno-modulatory role for VT in cattle. The verotoxin acts at two levels. First, VT enhances epithelial permissiveness and thus increases VTEC binding. Secondly, VT suppresses IL-8 synthesis and secretion thereby contributing to limiting a local inflammatory response. The sophistication and subtlety of VT

suggest that the bacteria have acquired these multifaceted molecules as an evolving feature to promote a hospitable niche within the environment of the bovine host.

5 Role of flagellaee in adherence of *E. coli* O157:H7 to bovine rectal epithelium

5.1 Introduction

Apart from LEE-encoded virulent determinants several other proteins have been proposed as novel adhesion factors for EHEC serotype O157:H7, including ToxB and Efa-1', required for expression of LEE-encoded type III secreted proteins (Stevens *et al.*, 2002b;Stevens *et al.*, 2004b); Iha, a 67 kDa outer-membrane protein similar to the product of *Vibrio cholerae* iron-regulated gene A, implicated in adherence to HeLa cells when expressed in *E. coli* K-12 (Tarr *et al.*, 2000); Cah, calcium-binding autotransporter protein expressed in *E. coli* DH5α enhanced bacterial auto-aggregation (Torres *et al.*, 2002b); OmpA, was associated with the hyper-adherent phenotypes in certain Tn*phoA* mutants during binding studies on HeLa cells (Torres and Kaper, 2003); and Saa, an autoagglutinating adhesin produced by LEE-negative strains (Paton *et al.*, 2001;Jenkins *et al.*, 2003a;Donnenberg *et al.*, 1993;Paton *et al.*, 2001). Analysis of the complete genome sequence of strain EDL 933, associated with an outbreak of haemorrhagic colitis in United States in 1982 (Perna *et al.*, 2001;Perna *et al.*, 1998) and strain RIMD 0509952 isolated from the Sakai outbreak (Hayashi *et al.*, 2001b)have identified 14 putative fimbrial gene clusters in *E. coli* O157:H7. Of these, roles for *lpf* and Loc 8 in colonization in cattle have been assigned (Dziva *et al.*, 2004). Long polar fimbriae (LPF) 1 and 2 expressed in non-fimbriated *E. coli* K-12 strain resulted in increased adherence to tissue culture cells (Torres *et al.*, 2002a;Torres *et al.*, 2004) and the O157 strains with mutated *lpfA1* and *lpfA2* poorly colonized sheep and pigs in experimental studies (Jordan *et al.*, 2004).

A role of LPS O-antigen side chains in adherence of O157:H7 STEC strains has also been examined (Bilge *et al.*, 1996;Cockerill, III *et al.*, 1996). In these studies Tn*phoA* mutagenesis was used to construct STEC strains deficient in O-antigen biogenesis, and these were found to be hyperadherent to Hep-2 cells *in vitro*. The enhancement of adherence might be due to increased exposure of OMPs on the bacterial surface,

although it might be due to the gross disturbance of cell surface hydrophobicity due to loss of 'O' antigen.

Although a substantial amount of data has been generated in recent years regarding the interaction of *E. coli* O157:H7 with host cells so far type III secretory proteins are the only O157:H7 virulence determinants demonstrated to play a role in intestinal colonization *in vivo.* However their role in intimate adherence is likely to be limited to later stages of infection (Donnenberg *et al.*, 1993;McKee *et al.*, 1995;Tzipori *et al.*, 1995;Donnenberg *et al.*, 1993) and factors conferring initial interaction of EHEC with intestinal epithelium remain to be clearly defined. EspA containing surface appendages were hypothesised to be important in initiating contact between EHEC and their target cells following which there was gradual reduction in these filaments and were later replaced by tighter attachment mediated by intimin (Ebel *et al.*, 1998b). Although the factors responsible may not be fully defined, it is clear that the molecular interactions of *E. coli* O157:H7 with intestinal epithelium are complex and multiphasic and likely involve multiple types of ligand-receptor contacts during the course of colonization.

Recently, evidence has been presented to support a role for *E. coli* flagellae in adherence to epithelium not merely via motility/chemotaxis but directly as an adhesin (Giron *et al.*, 2002). Specifically, purified H6 and H2 flagellae of EPEC bound human epithelial cells as assessed non-quantitatively by immunofluorescence and anti-H6 flagellae antibodies inhibited adherence of EPEC strain *E. coli* O127:H6 (E2348/69). The *fliC* mutant showed a 60% reduction in adherence and introduction of *fliC* gene from the EPEC strain into a K-12 strain conferred adherence reminiscent of localized adherence. Hence flagellae were implicated in pathogenicity of EPEC for which roles in initial adherence and microcolony formation were proposed. H2 flagellae are present on EHEC serotypes (e.g. O111:H2 & O113:H2) thus their contribution to adherence cannot be overlooked.

Flagellae interacts with the pattern recognition receptor (PRR) TLR5 located basolaterally (Gewirtz *et al.*, 2001a;Gewirtz *et al.*, 2001b;Gewirtz *et al.*,

2001b;Gewirtz *et al.*, 2001a), via conserved domains (Donnelly and Steiner, 2002) (Murthy *et al.*, 2004) resulting in activation of transcription factors (particularly NF-κB) leading to increased expression of, for example, antimicrobial peptides (Ogushi *et al.*, 2004) and other pro-inflammatory effectors such as IL-8 (Zhou *et al.*, 2003b) and CCL20 (Sierro *et al.*, 2001;Izadpanah *et al.*, 2001). Other epithelial (co-) receptors including TLR2 (Adamo *et al.*, 2004), gangliosides such as GM1, GD1a, and asialo-GM1 (McNamara *et al.*, 2001a;Feldman *et al.*, 1998b), and mucin MUC1 (Lillehoj *et al.*, 2004;Lillehoj, Kim, and Kim, 2002) have been proposed as receptors for flagellae of *Pseudomonas aeruginosa*. Gangliosides GD1a, GD1b, and GT1b acted as co-receptors with TLR5 for *Salmonella enterica* serovar *Enteritidis* flagellin (FliC) (Ogushi *et al.*, 2004) and therefore several complementary means of flagellae-epithelium interaction may operate. In addition to their involvement in inducing inflammation, a role in adherence of bacteria to epithelial cells has also been described for certain flagellae including those of *Clostridium difficile* (Tasteyre *et al.*, 2001), *Burkholderia pseudomallei* (Inglis *et al.*, 2003), *Aeromonas* spp. (Kirov, Castrisios, and Shaw, 2004a) and *Listeria monocytogenes* (Dons *et al.*, 2004).

On the basis of recent evidence on the role of flagellae as an adhesin the aim of this chapter was to examine the role of H7 flagellae in interaction of *E. coli* O157:H7 with bovine rectal epithelium (the principal site of colonisation in cattle) *in vitro*.

5.2 Materials and Methods

5.2.1 Bacterial strains

EHEC strains: ZAP 734 (Stx-negative *E. coli* O157:H7 strain NCTC 12900), ZAP 735 (*fliC*- isogenic mutant derived from strain NCTC 12900) were gifted by Prof. Martin Woodward); ZAP 196 (Stx-positive *E. coli* O157:H7 (Walla 1), ZAP 198 (Stx-negative *E. coli* O157:H7), ZAP 244 (O113:H21), ZAP 116 (O26:H11) and EPEC strain ZAP 286 (E2348/69, O127: H6).

5.2.2 Primary cell culture

Bovine primary epithelial cells were cultured as described in chapter 3. The cells were maintained in D-Valine medium supplemented with 1% or 2.5% batch-tested FCS, 0.25U/ml insulin, 10ng/ml EGF and 30μg/ml gentamicin. The cells were grown on collagen-coated 24-well culture plates or 4-well chamber slides (Costar, Corning USA) until confluence with approximately $3x10^5$ cells/well. It took approximately 10 to 14 days following primary epithelial cell culture to obtain a state of confluence.

5.2.3 Adherence assays

Adherence assays were performed on confluent cultures of bovine rectal primary epithelial cells. Overnight cultures of bacterial strains grown in MEM-HEPES with appropriate antibiotics (Streptomycin 25μg/ml, ZAP 735) were diluted 1:10 and further grown to an optical density of 0.3-0.4 at OD_{600} in a shaking incubator at 200 rpm at 37°C for approximately 3 h. Culture medium was removed and the confluent primary epithelial cells were washed twice in pre-warmed MEM-HEPES. The cells were infected at a multiplicity of infection (MOI) of 1:100 in MEM-HEPES at 37°C, 5% CO_2 for different time periods as described in each experiment. The infected cells were washed three times with PBS to remove the non-adherent bacteria, adherent bacteria were solublised/removed with PBS-0.1% (v/v) Triton X-100, serially diluted, and plated onto LB agar to determine the number of bacteria adhering to the cells in culture as colony forming units (cfu).

In adherence inhibition assay experiments, bacteria were pre-treated for 30 minutes at room temperature with rabbit anti-H7 polyclonal antibody (1:10) (Mast Diagnostics)

or cells were pre-incubated with purified H7 flagellin as potential binding inhibitors. In some experiments prior to infection of cells the bacteria were incubated in pre-conditioned medium (PCM) (see 5.2.12) for 1 h. In certain experiments as specified in the text immediately upon inoculation, mild centrifugation (1000rpm for 3 min) was applied to infected cells in 24-well tissue culture plates.

To test if induction of flagellae on contact with the bovine rectal epithelial cells is a general attribute to all the EHEC strains or is a unique feature of serotype *E. coli* O157:H7, adherence assays were done with a subset of EHEC O157:H7, O26:H11 and O113:H21 strains in a time course experiment (1 h and 3 h) and the expression of flagellae was examined in a non-quantitative immuno-fluorescence assay.

5.2.4 Statistical analysis

The raw data were transformed using logarithmic or square root transformations to ensure that the distributional properties of the data match those required for unpaired student's t-test. Statistics were calculated using GraphPad Prism® (version 3.0, GraphPad software, San Diego, CA, USA, www.graphpad.com), or Sigma Plot Scientific Graphic Software version 2.01. Results were considered significant if $p<0.05$.

5.2.5 Inhibition of ganglioside synthesis

A confluent culture of bovine rectal primary epithelial cells was incubated (37°C, 5% CO_2, 48 h) with or without 50 and 100 μm d,1-threo-1-phenyl-2-hexadecanoylamino-3-morpholino·HCl (PPMP) a glucosylceramide synthase inhibitor (Sigma) (Shayman *et al.*, 2000). The cells were then used for the adherence assays as above.

5.2.6 Immunofluorescence (IF) procedures

Following infection and thorough washing with PBS, the cells were fixed/ permeabilized with 2% (V/V) formalin/ 0.2% (v/v) Triton X-100. The specimens were prepared for IF as follows. Primary rabbit flagellaer-type specific or O-type specific antibodies (Mast Diagnostics) diluted 1:250 in PBS were added for 1 h. After washing, the cells were incubated for 1 h with secondary anti-rabbit IgG FITC/

TRITC-conjugated antibodies (Sigma) diluted 1:1000 in PBS. The cells were stained with TRITC-phalloidin (1μg/ml) (Sigma) and TO-PRO (Molecular Probes) for 20 minutes each. The cells were washed extensively, mounted in Fluoromount fluorescence mounting medium (DAKO) and examined using a Leica TCS NT confocal microscope.

To examine the binding of purified flagellae, bovine rectal primary epithelial cells were washed three times with pre-warmed MEM-HEPES and incubated with isolated flagellae 5 μg/ml (H7, H11 or H21) for 3 h at 37°C, 5% CO_2. The samples were processed for IF as above.

5.2.7 Image Acquisition procedures in confocal microscopy

Images were acquired using a Leica TCS NT confocal system (Leica Microsystems, GmbH, Heidelberg, Germany), equipped with an Argon/Krypton mixed gas laser, allowing 3-channel detection of fluorophores. Multi-channel images were acquired either simultaneously, or sequentially. A x63 Plan Apo oil immersion lens (NA 1.32) was routinely used, often in conjunction with the digital zoom capability of the confocal system.

As the size of bacteria is close to the limits of resolution of imaging systems, (approx 300 nm), it was decided to employ image restoration techniques to achieve optimal results. For this, data was acquired at Nyquist sampling rates, which is the minimum sampling distance required to ensure all information present in the sample is collected. These data sets were then deconvolved, using Huygens Professional (Scientific Volume Imaging b.v., Hilversum, The Netherlands), running on a Silicon Graphics computer. The resulting images showed a significant improvement on the clarity of the objects. While the visual improvement of the x-y image plane was noted, the main benefit of the deconvolution process was seen in the z-resolution of the dataset, which removed a large percentage of the distortion effect caused by the inherent Point Spread Function of the microscope system. This restoration of acquired data was recommended before further analysis of datasets, in particular, for any colocalisation analysis.

Information was required on possible colocalisation between the bacterial flagellae and cell surface. Deconvolution was routinely carried out on the experimental data sets acquired for this, before colocalisation analysis, using the Colocalisation module of Imaris (Bitplane AG, Zurich, Switzerland). This program analysed the complete 3D dataset, and reported the number of voxels colocalised and percentages of colocalisation between each channel in the data set.

5.2.8 *In Vitro* Organ Culture (IVOC) Assay

Tissue specimens obtained from adult cattle at a local abattoir were transported in ice cold HBSS (GIBCO BRL). The terminal rectal mucosa 3 cm proximal to the recto-anal juncton was carefully excised and washed in cold PBS, cut into 1cm squares with a thickness of 2 mm and placed in tissue culture medium RPMI 1640 (GIBCO BRL). The mucosal pieces were placed on a sterile foam pad immersed in pre-warmed (37°C) RPMI 1640 (Sigma-Aldrich). The bacterial cultures were grown as above for the adherence assays. The explants were infected with cultures of ZAP 734 or ZAP 735 strains (100μl) (as grown for the adherence assay) for 8 h at 37°C, 5% CO_2, 95% air in a humified atmosphere. After 2 h of infection the medium was replaced at every 1 hr interval. The infected tissue explants were given 3 washes in PBS, fixed and permeabilized overnight (4°C) in 4%(w/v) PFA/0.2% (v/v) Triton X-100, processed as whole mounts for IF studies as discussed previously.

5.2.9 Purification of flagellae

Overnight cultures of ZAP 734 (O157:H7), ZAP 116 (O26:H11) and ZAP 244 (O113:H21) grown statically at 37°C in LB broth were used to grow bacterial lawns on LB agar plates. The bacterial lawns were gently suspended in formyl saline (v/v, 0.4%). The flagellae were mechanically sheared by homogenization on ice (speed 3, 3 minutes) with a "whirling type blender (Power Gen 125, Fisher Scientific). Bacteria were removed by centrifugation (10,000g, 4°C, 30 minutes). Partially purified flagellae were recovered by ultracentrifugation (100,000 x g 4°C, 90 minutes) in a swinging bucket rotor centrifuge (model SW-40, Beckman, Durate, CA

Beckman). At this stage a part of crude flagellum preparation was fixed in 3% gluteraldehyde and examined by electron microscopy. The pellet was suspended in caesium chloride solution (1.3 g cm^{-3} density) and centrifuged at (100,000g 4°C, 21 h) in a swinging bucket rotor centrifuge. Flagellae formed an opaque band at a refractive index of 1.3630 that was collected with a 26-gauge needle into a 1ml syringe. The purified flagellae were resuspended in PBS and pelleted by ultracentrifugation (100,000g 4°C, 90 minutes) to remove the caesium chloride. The purified flagellae pellet was resuspended in sterilized distilled water and aliquots were kept at -20°C. A mock flagellae preparation was prepared in an identical manner using ZAP 735 (*fliC* mutant) as a control.

Protein concentrations were determined using DC Protein Assay kit Bradford as per the manufacturer's instructions, using bovine serum albumin as standard (Bio-Rad, Richmond, CA).

5.2.10 Electron microscopy

Strains (ZAP 734 and ZAP 735) were grown overnight at 37°C in LB broth under static conditions, suspended in phosphate-buffered saline (PBS, pH 7.4), fixed in 3% gluteraldehyde and allowed to adhere to Formvar-carbon-coated copper grids (200 mesh, Electron Microscopy Sciences). The expression of flagellae on these strains or isolated flagellae were visualized by negative staining with 2% potassium–phosphotungstic acid, pH 6.8 and the grids analyzed in a Phillips 201 electron microscope. To visualise adherent bacteria the bovine primary rectal epithelial cells were cultured on Thermanox coverslips. The infected monolayers were fixed in 3% glutaraldehyde and processed for scanning electron microscopy as described before in chapter 2.

For immunogold labelling of flagellae, the infected cells were fixed in 2% Formalin/0.25% gluteraldehyde for 30 minutes at RT. The cells were washed twice in PBS and reacted with anti-H7 and/or anti-O157 antiserum followed by 10-nm gold-labelled anti-rabbit IgG (British Biocell International) for 30 minutes each at room temperature. The immuno-gold labelled specimens were fixed in 4% gluteraldehyde and further processed as described in chapter 2. The specimens were

examined using secondary and back scattered electron detection system in Hitachi 4700 Field Emission Scanning Electron Microscope.

5.2.11 SDS-PAGE and immunoblot analysis

The flagellae preparations were adjusted to the same protein concentration and then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were stained with colloidal blue or transblotted to Immobilon-P membranes (Millipore Corp., Bedford, Mass). The membrane was blocked overnight with blocking buffer (PBS-Tween 20 (0.1%)-BSA 3%) at 4°C, washed twice with PBS-Tween 20 (0.1%) and reacted with rabbit antisera specific to flagellin type (H7, H11 or H21) (Mast Diagnostics) diluted 1:1,000 in blocking buffer for 2 h. After the membrane was washed for 2 h, bound antibody was detected on incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako A/S, Denmark) diluted 1:3,000 in blocking buffer for 1hr and developed in enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Arlington Heights, IL).

Protein characterisation: After SDS- PAGE the separated band was excised from gels and analysis was done by the Functional and Genomics Unit, Moredun Research Institute. Briefly, proteins were destained and reductively alkylated using DTT and Iodoacetamide. The gel pieces were then digested overnight with Trypsin (Promega Porcine trypsin) at 37°C. Digests were analysed on a Voyager DE-PRO MALDI-ToF mass spectrometer (Applied Biosystems), scanning the 600 to 5000 dalton region in reflectron mode producing monoisotopic resolution. The spectra generated were mass calibrated using known standards and the peaks deisotoped. Masses obtained were then database searched using the MASCOT search engine and the NCBInr and Swissprot databases. Searches were conducted using 50 ppm and 100 ppm mass tolerance windows.

5.2.12 Preconditioned medium

Confluent cultures of primary bovine rectal epithelial cells that had been washed thrice with pre-warmed (37°C) MEM-HEPES were incubated with MEM-HEPES (Sigma) without antibiotics or fetal bovine serum for 24 h. The supernatant referred as to 'preconditioned medium' (PCM) was filtered through a 0.2 μm membrane and

aliquots stored at -20°C. PCM was used for pre-incubation of EHEC strains in certain adhesion assays or for examination of expression of flagellae.

5.2.13 Quantification of H7 expression

Overnight cultures of ZAP 734 grown in MEM-HEPES at 37°C in a shaking incubator (200 rpm) were diluted 1:10 in MEM-HEPES and grown to an optical density of 0.3 to 0.4 at OD_{600}. An aliquot (100 μl) of bacterial culture was incubated at 37°C with 400 μl of PCM or MEM-HEPES for 1 h. The bacterial cells were then fixed and permeabilized with 4% (v/v) Formalin/ 0.1%(v/v) Triton X-100 for 20 minutes and then stained with rabbit anti-H7 polyclonal followed by FITC-labelled goat anti-rabbit antibody. An aliquot (100 µl) of stained sample was thoroughly resuspended and diluted into 1 ml of PBS. Samples were left to equilibrate for 5min at room temperature then data acquired at the flow cytometer. The flow cytometer used was FACSCalibur (Becton Dickinson, NJ, USA) equipped with an Argon-ion laser emitting at 488 nm and Cell Quest software. Forward (size) and side scatter signals were collected in logarithmic scale. FITC signal was collected with a 530/30 band pass filter in logarithmic scale. An acquisition gate was drawn around the bacterial population in Forward and Side scatter, to exclude debris and background noise. A minimum of 10000 gated events were acquired for each sample. Data was analyzed using CellQuest software. For each sample a bi-parametric fluorescence dot plot was generated (FL1-corresponding to FITC) and the percentage of positive cells for the specific stain was calculated by the software. Moreover, fluorescence histograms were generated (FL1 vs number of cells) and different samples overlayed to compare fluorescent profiles. Experiments were repeated three times.

All the above experiments were repeated at least three times to confirm the results obtained. Unless otherwise indicated, the results shown here are representative of a particular assay or observation under the microscope.

5.2.14 Dot blot binding assay

The binding of different purified flagellin (H7, H11, H21) from different EHEC serotypes to asialo-GM1 (Sigma) was tested. Aliquots (2 μl) of asialo-GM1 and 700 μg of each of purified flagellin as positive controls were applied to three Immobilon-P membranes (Millipore Corp., Bedford, Mass). Specific controls were employed to test the non-specific binding of primary and secondary antibodies. The membranes were dried at 37°C for 15 minutes and then blocked overnight in 3% BSA–PBS 0.1%Tween-20 solution at 4°C on a shaking platform. After two washes with PBS-0.1%Tween-20 (30 minutes), the membranes were incubated overnight with 700 μg of specific purified flagellaer suspension in PBS-0.1% Tween-20 at 4°C on a shaking platform. After three washes with PBS-0.1% Tween-20 (30 minutes) membranes were immuno-stained and developed as described previously.

5.3 Results

5.3.1 *E. coli* O157 lacking flagellae exhibit diminished adherence to bovine primary rectal epithelial cells

To examine the possible role of H7 as an adhesin, the adherence of wild-type *E. coli* O157:H7 flagellaete strain ZAP 734 (NCTC 12900), and the isogenic *fliC* mutant ZAP 735 to bovine rectal primary epithelial cells was compared. The phenotypes of these strains were confirmed: ZAP 734 when grown in LB expressed flagellae and was motile in LB motility agar (Fig. 5.1A, C) whilst the *fliC* mutant ZAP 735 was non-motile and showed no expression of flagellae (Fig. 5.1B, D).

At 3 h the aflagellaete ZAP 735 strain adhered sparsely (Fig. 5.1F) and expressed no flagellae compared to wild type ZAP 734 which showed localized adherence (Fig. 5.1E), with abundant expression of flagellae (Fig. 5.1G) and substantial microcolony formation. Quantification of colony forming units (cfu) revealed that wild type

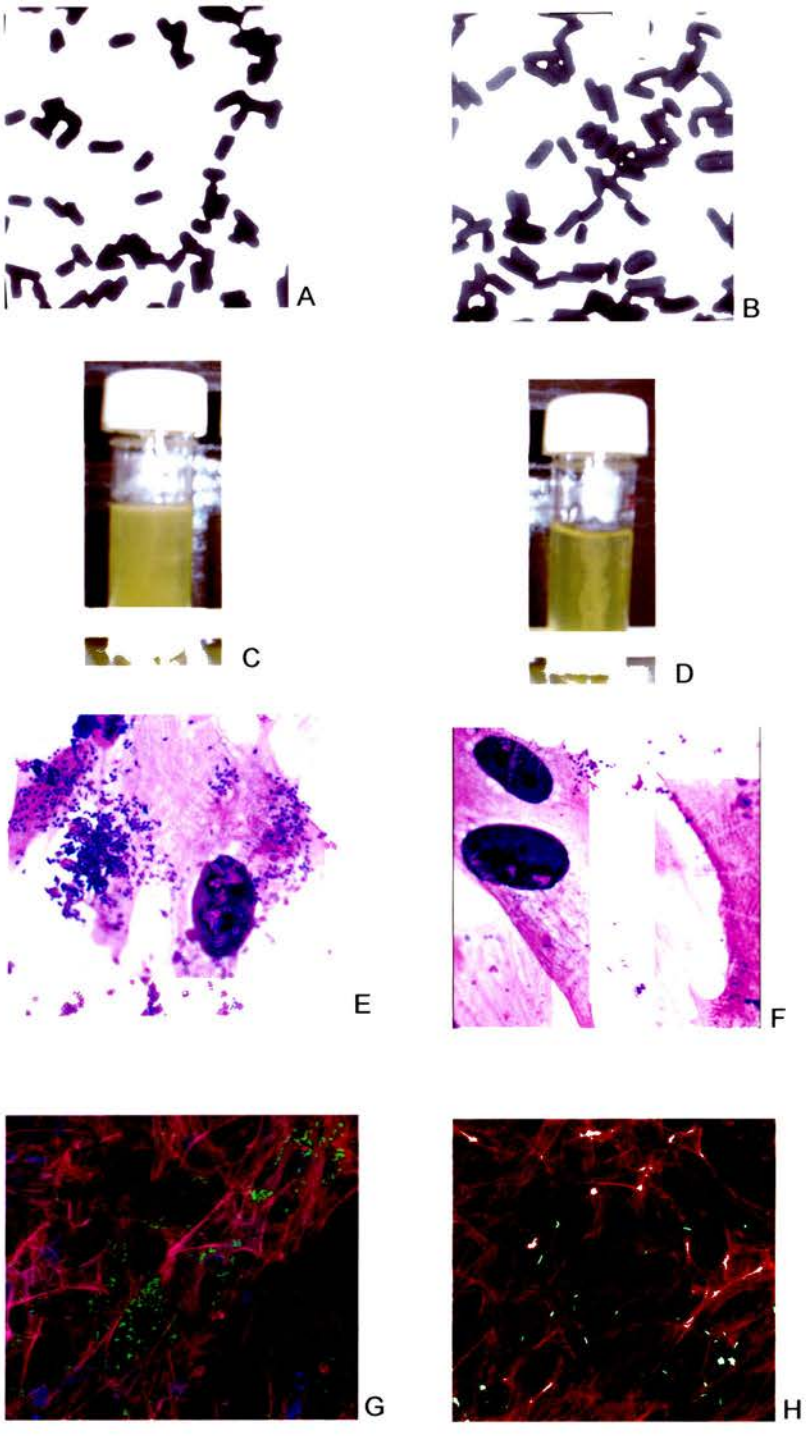

Fig. 5.1 Phenotypes of the wild type *E. coli* O157:H7, ZAP 734 (NCTC 12900) and the isogenic *fliC*-mutant ZAP 735. Expression of flagella was assessed by transmission electron microscopy. ZAP 734 expressed flagella (A) however the mutant ZAP 735 did not (B). Motility Test for ZAP 734 and ZAP 735. Glass vials containing LB supplemented with 0.3% agar and were stab inoculated with bacterial cultures and incubated for 16-18 h at 37°C. The motility was visualised as uniform diffusion around the line of inoculation. ZAP 734 is highly motile (C) in comparison to aflagellate ZAP 735 (D). Diff-Quik (E-F) and immunofluorescent stained (G-H) photomicrographs showing adherent phenotype of ZAP 734 and ZAP 735. At 3 h post-infection wild type ZAP 734 showed localized adherence (E) and most of the adherent bacteria expressed flagella - seen as wavy green filaments (G). The aflagellate mutant strain ZAP 735 showed sparse adherence (F) without expression of flagella (H). For immunofluorescencec test cells were stained with actin specific phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) conjugated antibody using a Leica TCS NT confocal system (x 63 objective lens).

ZAP 734 adhered significantly higher than the *fli*C mutant ZAP 735 at 1 hr (p=0.0001) and 3 hr (p=0.0001) post infection, respectively (Fig. 5.2).

To overcome any anomalies of initial cell-bacterium interaction, i.e to determine whether this reduction in initial adherence of *fliC*-mutant was due to loss of motility, binding assays were carried out in which bacterial cells were centrifuged onto bovine rectal primary epithelial cells and, after a short incubation of 15 minutes, adherent bacteria were enumerated. The mild centrifugation significantly enhanced binding of both the flagellaete wild type ZAP 734 and isogenic *fli*C mutant ZAP 735 strains (p<0.0001). Importantly, following centrifugation the wild type flagellaete ZAP 734 strain still adhered significantly more than the aflagellaete mutant (Fig. 5.3) (p<0.0003).

5.3.2 *E. coli* O157 lacking flagellae exhibit diminished adherence to tissue explants from terminal rectal mucosa

To examine the role of flagellae in binding of *E. coli* O157:H7 to bovine gut, *in vitro* organ culture technique was used. The explants from terminal rectal mucosa were infected with wild-type *E. coli* O157: H7 flagellaete strain (ZAP 734) which formed large and compact microcolonies (Fig. 5.4A) in comparison to the isogenic *fli*C mutant strain (ZAP 735) which exhibited sparse adherence and only occasional microcolonies (Fig. 5.4B). Some of the wild-type bacteria that could be seen expressing flagellae where present as single cells but not as microcolonies.

5.3.3 Flagellae antiserum inhibits *E. coli* O157:H7 binding to Bovine rectal primary epithelial cell

To confirm the role of H7 in adherence, inhibition assays were done. Wild-type ZAP 734 and the isogenic *fli*C mutant ZAP 735 were treated with anti-H7 antibody (1:10) for 30 min prior to infection of cells, and after 1 h incubation the adherent bacteria were enumerated as cfu counts per ml. As shown in Fig. 5.5, H7-antiserum significantly inhibited the binding of wild type flagellaete strain ZAP 734 (p=0.0039) but had no effect on the the *fli* C mutant ZAP 735 (p=0.86).

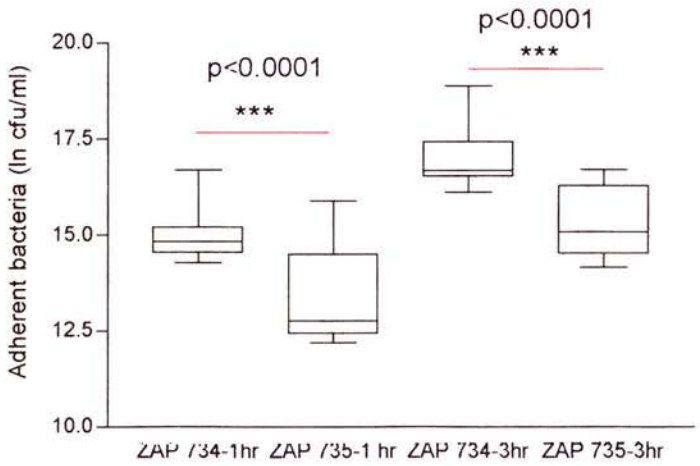

Fig. 5.2 Adherence of wild type *E.coli* O157:H7 strain ZAP 734 and the isogenic *fliC* mutant ZAP 735 to bovine rectal primary epithelial cells. The cells were infected at MOI 1:100 with bacterial cultures in mid-log phase of growth at 37°C, 5% CO_2. Adherent bacteria were solublised with PBS-0.1% Triton X-100, serially diluted, and plated to enumerate as colony forming units (cfu). ZAP 734 adhered significantly higher than the aflagellate strain at 1 h and 3 h post infection. Unpaired student's t-test was used to compare the groups statistically. The data were plotted using a box and whiskers representation. The box extends from the 25th to 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value and up to the largest.

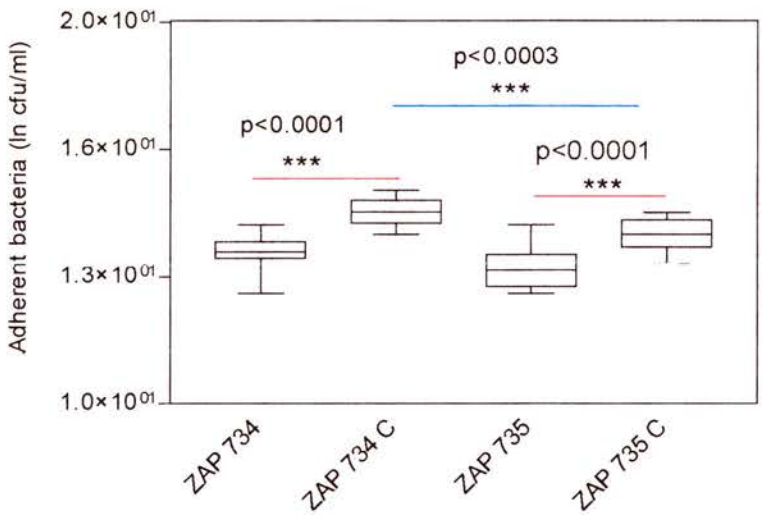

Fig. 5.3 Effect of mild centrifugation on adherence of wild type *E. coli* O157:H7 strain ZAP 734 and the isogenic *fliC* mutant ZAP 735. The cells were infected at MOI 1:100 with bacterial cultures in mid-log phase of growth at 37°C, 5% CO_2. Mild centrifugation (1000rpm, 3min) was applied to infected cells in tissue culture plates immediately upon inoculation and after 15 min of incubation the adherent bacteria were enumerated as colony forming units (cfu). Centrifugation significantly enhanced adherence of both flagellate ZAP 734 and aflagellate ZAP 735 ($p \leq 0.0001$) but the effect of centrifugation (C) was significantly more on ZAP 734 C than the ZAP 735 C ($p \leq 0.0003$). The groups were compared statistically using unpaired student's t-test.

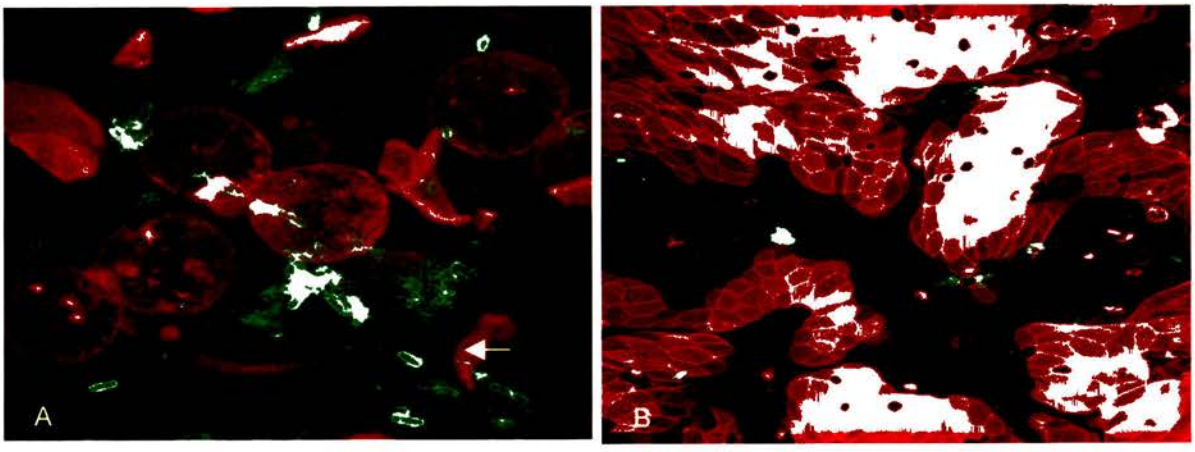

Fig. 5.4 Confocal micrograph showing binding of ZAP 734 (*E. coli* O157:H7 NCTC 12900 strain) and the isogenic *fliC* mutant strain ZAP 735 to the tissue explants from most terminal rectum of cattle in *in vitro* organ culture assay. The tissue explants were infected with bacterial cultures in mid-log phase of growth at 37°C, 5% CO_2 and 95% air in a humified chamber. The infected tissue explants were fixed/permeabilized in 4%(w/v) PFA/0.2%(v/v) Triton X-100 and stained with Phalloidin-TRITC (red). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) conjugated antibody. The tissue explants were microscopically examined as whole tissue mounts using a Leica TCS NT confocal system (x 63 objective). At 8 h post-infection the wild type ZAP 734 formed compact microcolonies. Some of the bacteria could be seen expressing the flagella (arrow) (A). ZAP 735 (*fliC* -) exhibited sparse binding (B).

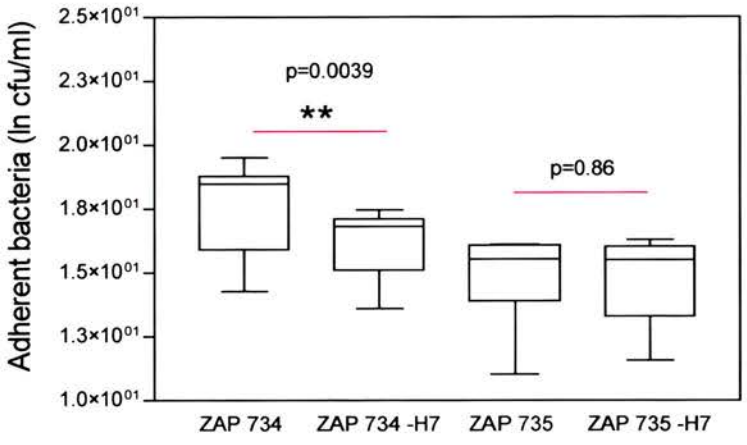

Fig. 5.5 Binding of *E. coli* O157:H7 to bovine rectal epithelial cells is inhibited by type specific flagellin anti-serum. Wild type flagellate strain ZAP 734 and isogenic *fliC* mutant ZAP 735 in mid-log phase of growth were pre-treated with anti-H7 antibodies (1:10) at room temperature for 30 min before infecting the cells at approximate MOI 1:100 at 37°C, 5% CO_2 for 1 h. Adherent bacteria were solublised with PBS-0.1% (v/v) Triton X-100, serially diluted, and enumerated as colony forming units (cfu). In presence of anti-H7 polyclonal antisera the binding of flagellate strain (ZAP 734-H7) was significantly decreased (p=0.0039) with no significant effect on the aflagellate mutant (ZAP 735-H7). Unpaired student's t-test was used to compare the groups statistically (n=2).

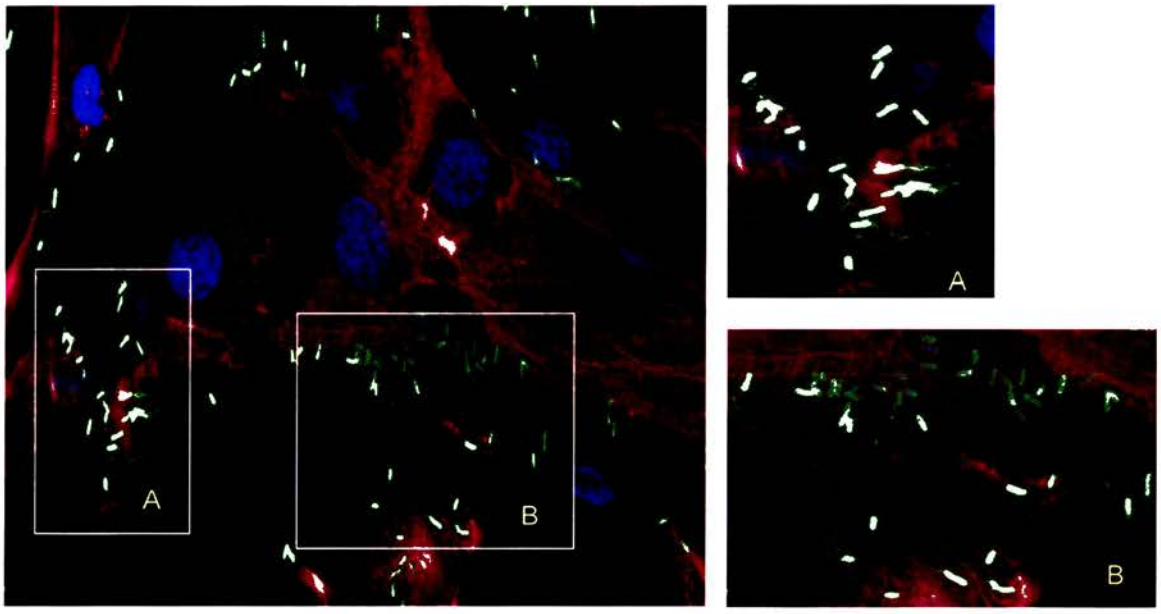

Fig. 5.6 Detection of flagella produced by ZAP 196 (*E. coli* O157:H7, Walla 1) Stx+ strain on binding to bovine rectal primary epithelial cells. The cells were infected at MOI 1:100 with bacterial culture in mid-log phase of growth at 37°C, 5% CO_2. At 1 h post-infection most of the bacteria expressed flagella - seen as wavy green filaments. Flagella from adjacent bacteria were seen forming intercalating bridges. Right hand panels show higher magnification images of regions indicated in main image. Cells were stained with actin specific phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) conjugated antibody and examined using a Leica TCS NT confocal microscope (X63 objective lens).

5.3.4 Demonstration of flagellae on adhering bacteria

Wild-type *E. coli* O157:H7 strains upon adherence to bovine rectal primary epithelial cells expressed flagellae abundantly (Fig. 5.6). In these IF micrographs flagellae can be seen as wavy filaments forming a web-like meshwork on the cell surface. The flagellae formed an extensive network between the adjacent bacteria and possibly mediated direct interaction with the epithelial cells. In co-localization (dual labelling) studies, flagellae could be seen contacting directly the epithelial cell (Fig. 5.7). Confocal microscopy clearly demonstrated what appeared to be contact points between flagellaer filaments and the apical surface of epithelial cells (Fig. 5.7D1, D2).

Interaction of flagellae with infected bovine rectal epithelial cells was further examined by scanning electron microscopy (SEM). The wavy filament resembling flagellae can be seen intercalating within the micro-villi (Fig. 5.8 A-B). The identity of the wavy structures observed was confirmed to be flagellae by immunogold labelling and high resolution field SEM using anti-H7 antibodies and anti-rabbit IgG conjugated 10nm gold particles (Fig. 5.8 C-D). At the contact points with the epithelial cell the flagellaer surface was not apparently available for the IG, seen as unlabelled intercept on the uniformly labelled flagellae (Fig. 5.8 E-F). Together these microscopic observations support the role of H7 as a possible adhesin to bovine rectal epithelial cells.

5.3.5 Temporal expression of flagellae during adherence

Initial observations at 3 h post-infection showed flagellae expression by individual or small clusters of *E. coli* O157:H7 but not larger compact groups or microclonies. This may occur through changes in expression of flagellae during infection therefore temporal expression of flagellae during adherence to bovine rectal primary epithelial cells was examined. Adherence assays were done using two wild-type EHEC strains (ZAP 193 and ZAP 196), over a time course and the expression of flagellae was examined by IF. After 1 h of infection the majority of individual adherent bacteria expressed flagellae (Fig. 5.9A, C). After 3 h of infection bacteria formed visible micro-colonies and at this stage only few of the adherent bacteria in the micocolony

expressed flagellae (Fig. 5.9B, D). At 8 h post-infection adherent bacteria formed typical A/E lesions characterised by actin-pedestals. The pedestal-associated bacteria

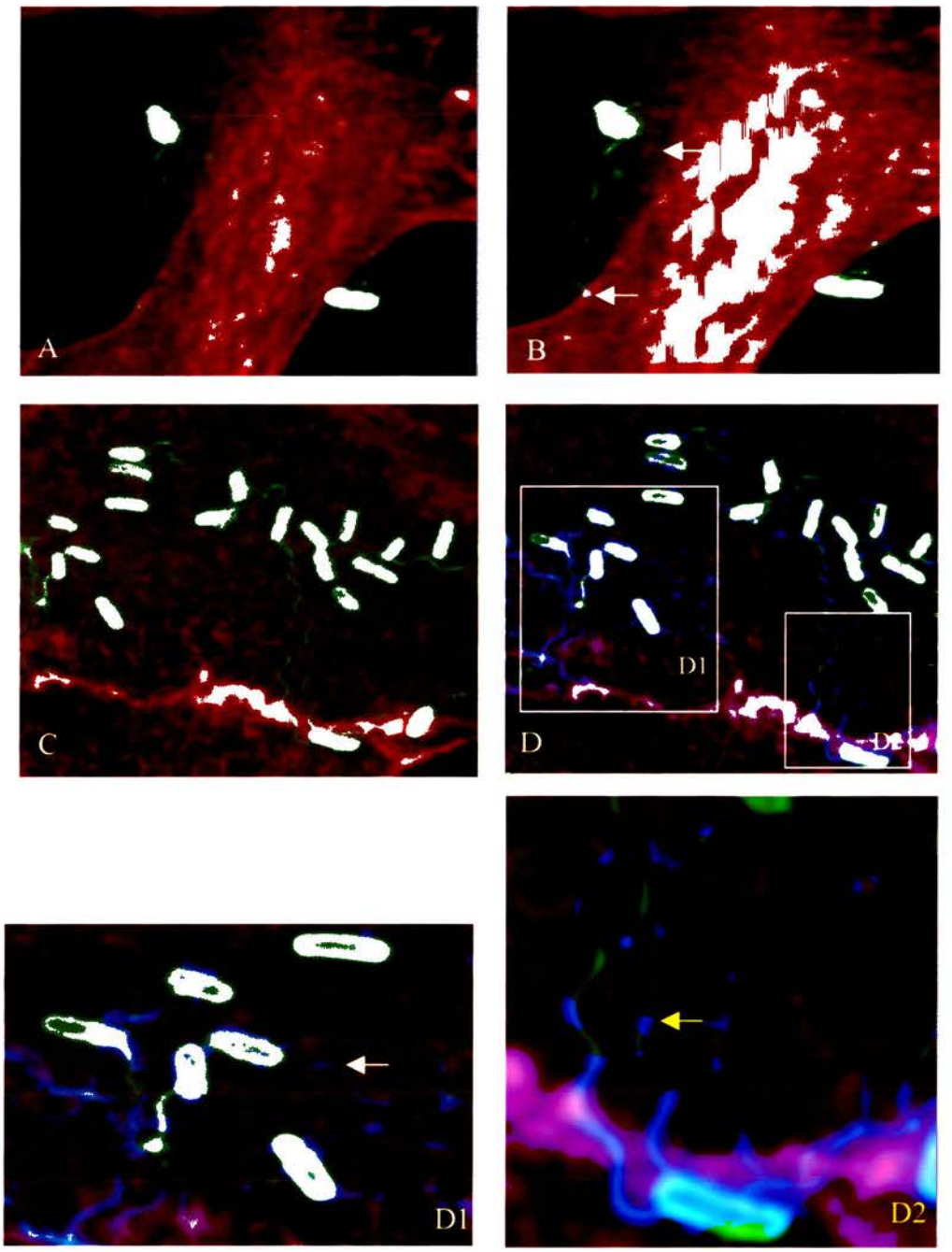

Fig. 5.7 Demonstration of H7 flagella as adhesin. Micrographs of ZAP 196 (*E. coli* O157:H7, Walla 1) adhering to bovine rectal primary epithelial cells. The cells were infected at MOI 1:100 with bacterial culture in mid-log phase of growth at 37°C, 5% CO_2 for 1 h. Flagella is seen tethered to the epithelial membrane as an initial contact (A) - clear contact points are marked with arrows (B). The adherent bacteria expressed flagella in abundance, seen as wavy filaments (C). The bacteria could be seen using the flagella to bind to the cell surface (D) - contact points are marked with arrows in magnified images (D1,D2). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) conjugated antibody. The cells were stained with phalloidin-TRITC (red). The 0.2 μm thick optical sections of the image were acquired sequentially using Leica TCS NT confocal microscope (x 63 objective lens). Images (D1 & D2) were digitally magnified by a factor of 6. The co-localization studies were done using "Colocalisation module of Imaris" which reports number of voxels colocalised and percentages of colocalisation between each channel (red and green) in the dataset, seen as blue in images (B, D, D1 and D2).

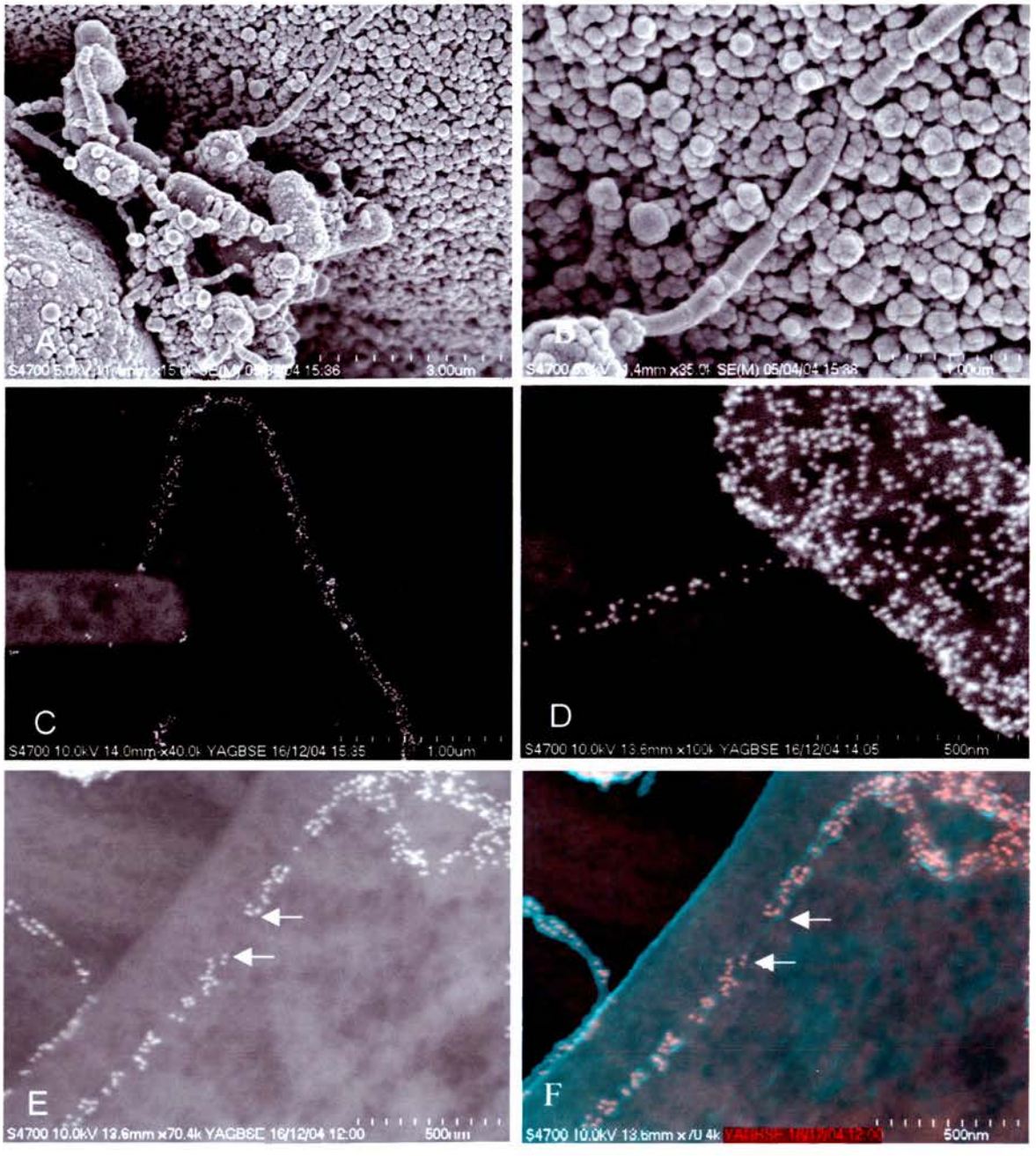

Fig. 5.8 High resolution SEM of *E. coli* O157:H7 (ZAP 196) adhering to bovine rectal primary epithelial cells. Filamentous structures resembling typical flagella can be seen intercalating with the microvilli on the apical surface of the cell (A, B). Immunogold (IG) labelling of flagella with anti-H7 antibodies (C) and of bacterium with both anti-O157 and anti-H7 antibodies (D) demonstrated the aggregation of 10nm gold particles to the 50-nm-wide wavy filament. The flagellum is uniformly labelled with gold particles except where it is possibly embedded in the epithelial cell thus not available for the IG binding (pointed with arrows) (E) as also shown in the combined back scattered and secondary electron image with pseudocoloured gold particles (F).

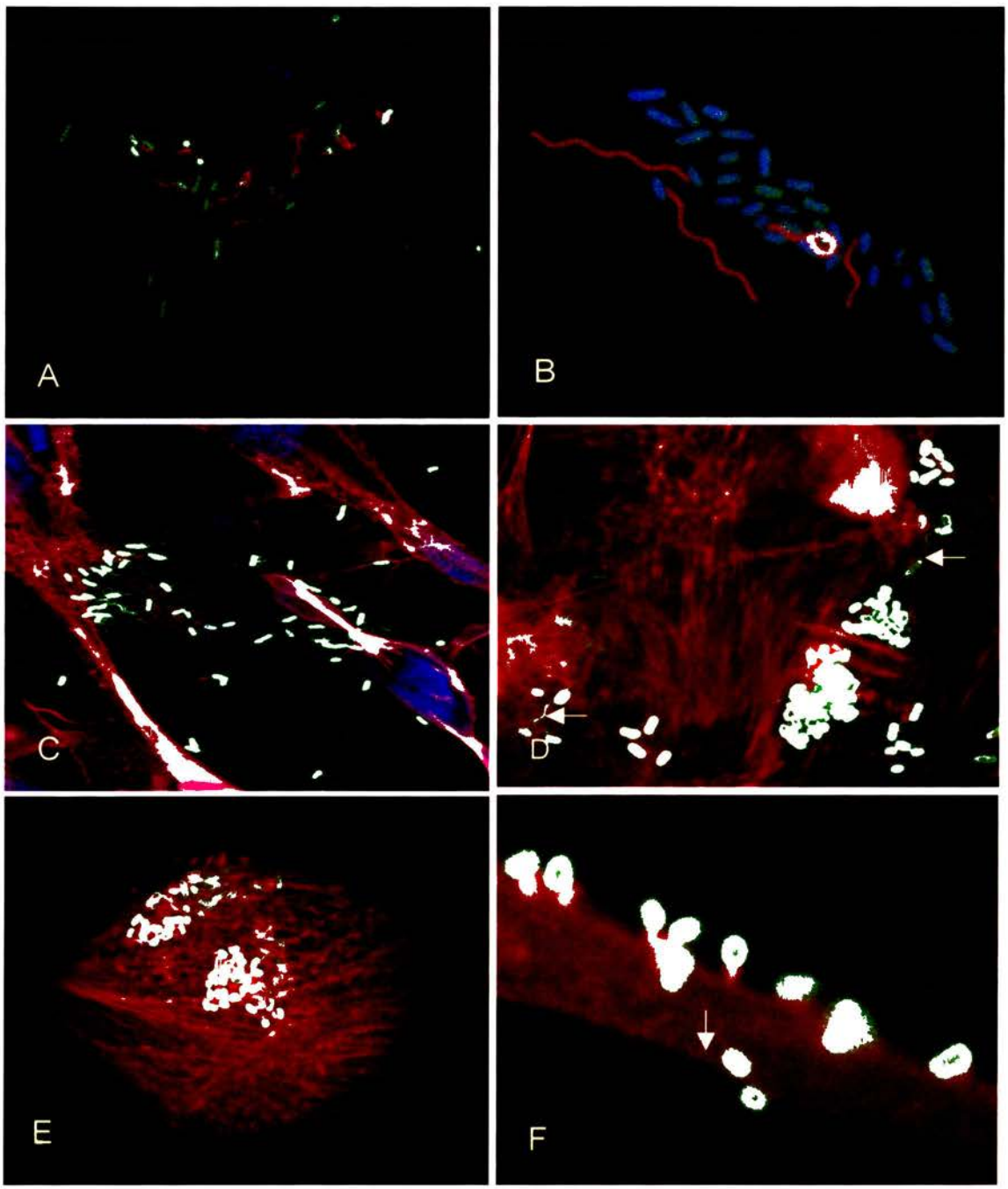

Fig. 5.9 Temporal expression of flagella by different wild type *E. coli* O157:H7 ZAP 193 (A-B) and ZAP 196 (C-F) strains on binding to bovine rectal primary epithelial cells. The cells were infected at MOI 1:100 with bacterial culture in mid-log phase of growth at 37°C, 5% CO_2. At 1 h post infection most of the adherent bacteria expressed flagella (A-C) and at 3 h the flagellar expression was limited to only few adherent bacteria (arrow) but rarely by those in the microcolony (B-D). At 8 h post-infection the bacteria associated with actin-pedestals forming A/E lesion did not show expression of flagella (E-F) although the bacterium associated with cells, but not with actin-pedestals, was still flagellated (arrow) (F). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) or TRITC (red) conjugated antibody. The cells were stained with phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The images were captured using a Leica TCS NT confocal microscope (x 63 objective lens).

were non-flagellaeted (Fig. 5.9E, F), whereas the bacteria associated with cells, but not with actin-pedestals, were still flagellaeted (Fig. 5.9 F).

5.3.6 Expression of flagellae by different EHEC strains

To test if induction of flagellae on contact with the bovine rectal epithelial cells is a general attribute to all the EHEC strains, adherence assays were done with a subset of EHEC serotypes and the expression of flagellae was examined by IF at 1 hr and 3 h time points. Both ZAP 193 and ZAP 196 strains expressed flagellae and later formed compact microcolonies at 1 h and 3 h, respectively (Fig. 5.10A-D). Bacteria in microcolonies did not express flagellae. ZAP 116 (O26:H11) and ZAP 244 (O113:H21) did not express flagellae at either time point examined (Fig. 5.10E-F) and apparently adhered poorly in comparison to ZAP 196 and ZAP 193, though it was not quantified.

5.3.7 The expression of H7 in *E. coli* O157 is triggered by a secreted epithelial component

The expression of microbial virulence factors (including adhesins) is triggered by various environmental cues. Recent publications (Sperandio *et al.*, 2003;Vlisidou *et al.*, 2004;Chen *et al.*, 2003;Lyte *et al.*, 1997) have shown that host-derived signals in the form of secretory molecules are important factors at the interface of host-pathogen interaction. To examine the effect of secretory signals from the eukaryotic cell on adherence, the wild type *E. coli* O157 strain ZAP 734 and the isogenic aflagellaete mutant ZAP 735 were incubated either in pre-conditioned medium (PCM) or MEM-HEPES before doing the binding studies. Pre-incubation in PCM significantly enhanced binding of ZAP 734 ($p=0.0006$) compared with ZAP 735 ($p=0.7920$) (Fig. 5.11). To further investigate if this increase in binding was an effect of enhanced expression of flagellae, strain ZAP 734 was incubated with PCM or MEM-HEPES and compared for the expression of H7 using Flow cytometry. PCM in comparison to MEM-HEPES markedly increased the expression of flagellae (Fig. 5.12). Thus, biosynthesis of H7 flagellae is triggered by bioactive molecule(s) secreted by bovine rectal primary epithelial cells and it is concluded that the higher expression of flagellae contributed to the subsequent enhanced binding of ZAP 734.

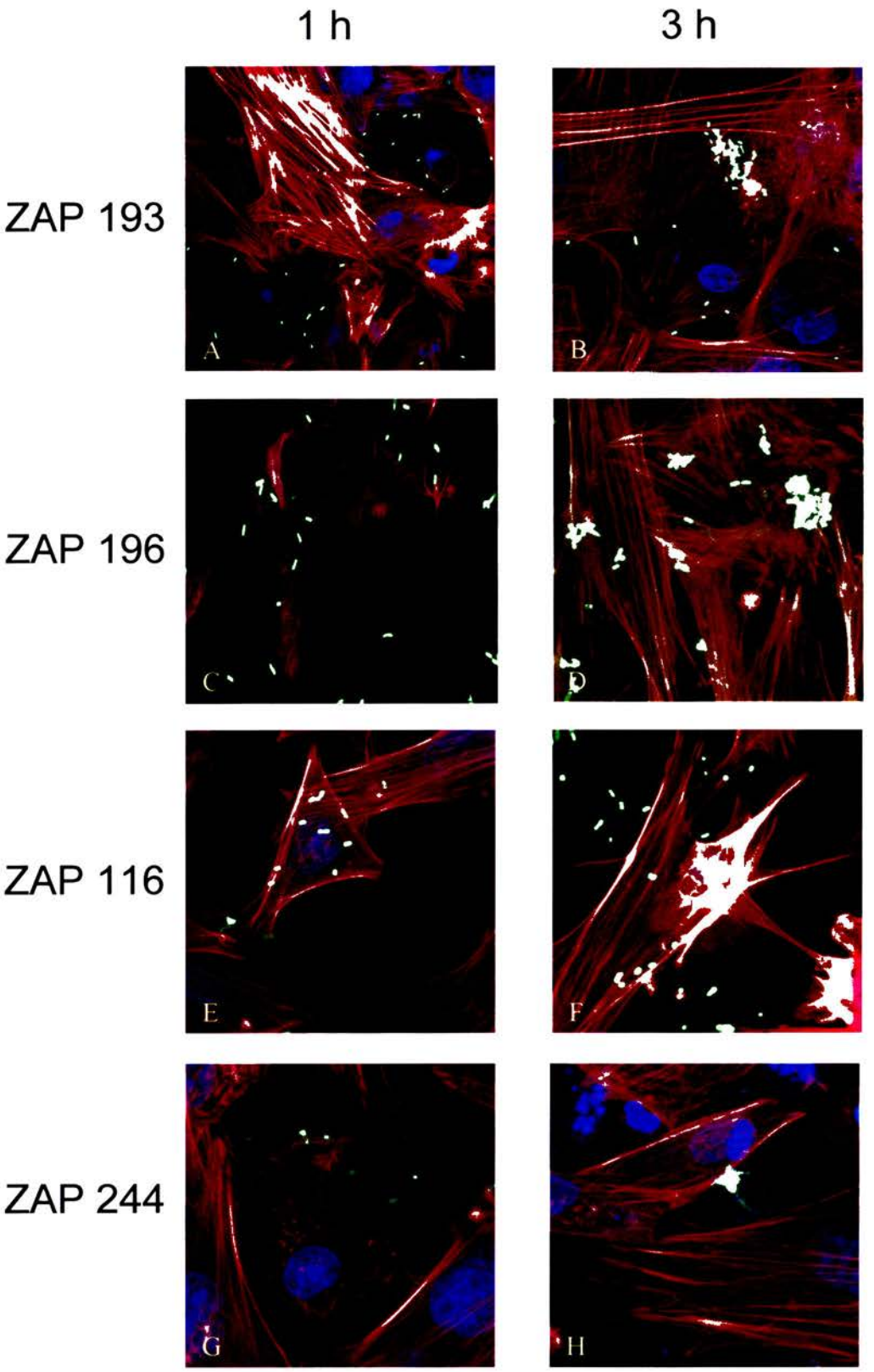

Fig. 5.10 Expression of flagella by different EHEC serotypes on adherence to bovine rectal primary epithelial cells. The cells were infected at MOI 1:100 with bacterial cultures in mid-log phase of growth at 37°C, 5% CO_2 for 1 h and 3 h. The infected cells were fixed/ permeabilized and stained with actin specific phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The bacteria and flagella labelled with O-type and flagella type-specific antibodies were detected with secondary FITC (green) conjugated antibody. The images were captured using a Leica TCS NT confocal microscope (x 63 objective lens). At 1 h both *E. coli* O157: H7 strains ZAP 193 (A) and ZAP 196 (C) expressed flagella but not *E. coli* O26:H11 (ZAP 116) (E) and *E. coli* 113:H21 (ZAP 244). At 3 h ZAP 193 (B) and ZAP 196 (D) formed compact microcolonies and individual adhered bacteria expressed flagella; ZAP 116 (F), ZAP 244 (H) adhered sparsely and did not express flagella.

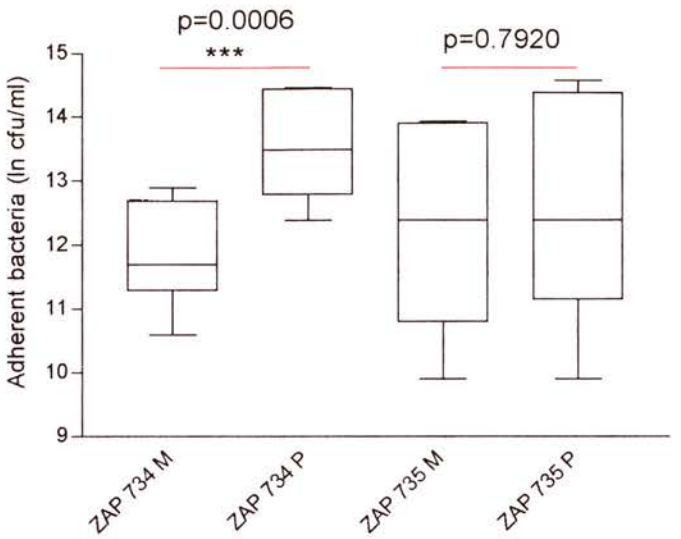

Fig. 5.11 Effect of Pre-conditioned medium (PCM) on adherence of wild type *E. coli* O157:H7 strain ZAP 734 and the isogenic *fliC* mutant ZAP 735 to bovine rectal primary epithelial cells. The bacterial cultures in mid-log phase of growth were pre-incubated with PCM (P) and MEM-HEPES (M) for 1h at 37°C prior to infection. The cells were infected at approximate MOI 1:100, at 37°C, 5%CO_2 for 1 h. Adherent bacteria were solublised with PBS-0.1% (v/v) Triton X-100, serially diluted, and plated to enumerate as colony forming units (cfu). PCM treatment significantly enhanced adherence of ZAP 734 than ZAP 735. Unpaired student's t-test was used to compare the groups statistically. (n=3)

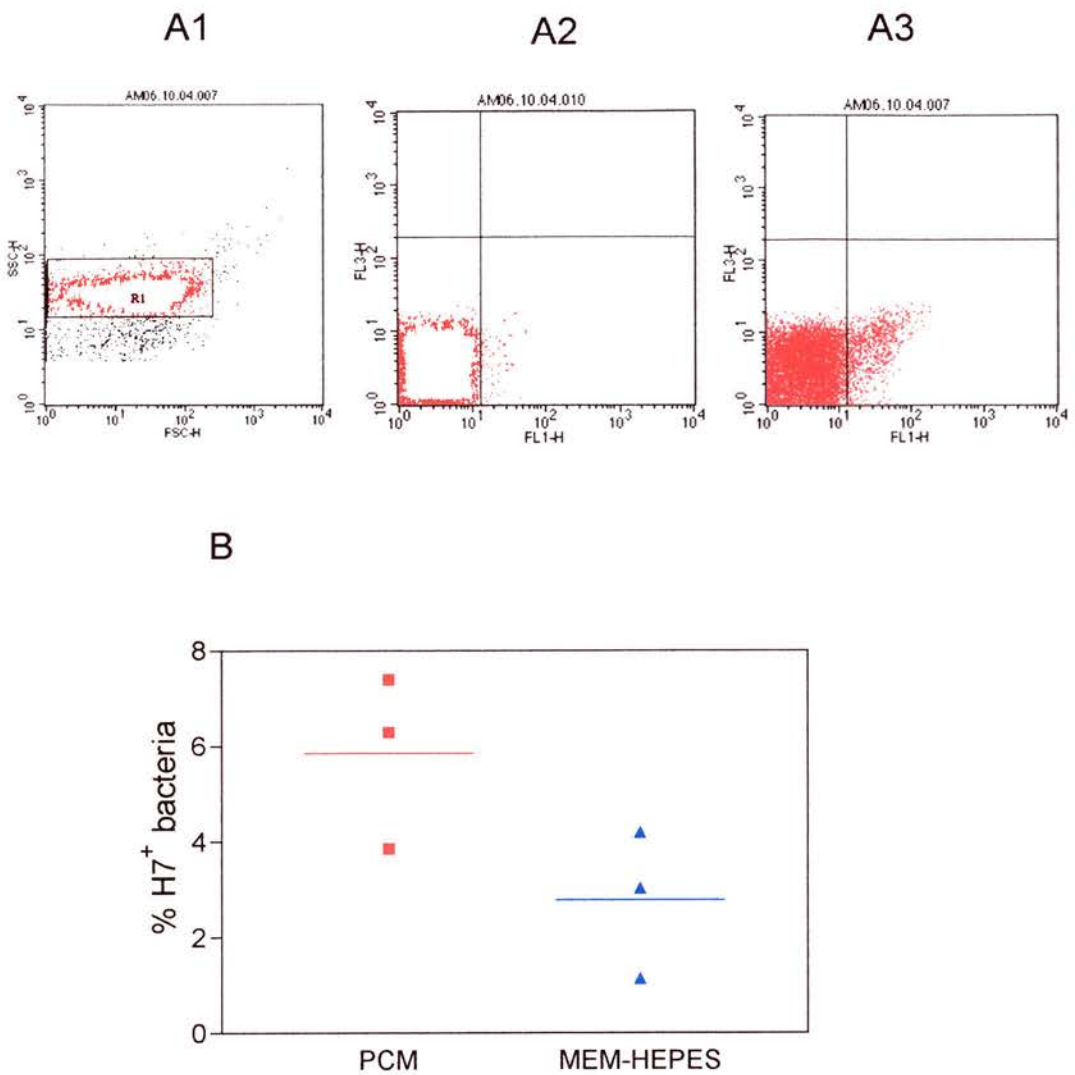

Fig. 5.12 Pre-conditioned medium (PCM) treatment enhanced expression of flagella by wild type *E. coli* O157:H7 strain ZAP 734. The bacterial cultures in mid-log phase of growth were incubated with PCM and MEM-HEPES for 1h at 37°C. The bacterial cells fixed/permeabilized (4%PFA/ 0.1% Triton X-100) were stained with primary antibody against H7 followed by FITC-labelled secondary antibody (x-axis). The expression of H7 flagella was then analysed by flow cytometry. (A) Representative FACS plots showing the expression of H7 flagella from one experiment. A1 depicts the gating on bacterial population. Bacteria expressing flagella in MEM-HEPES (A2) and PCM (A3) are depicted. (B) The experiment was done thee times and each point represents an individual experiment.

5.3.8 Flagellae possess adhesive properties

On the basis of the above data, it was speculated that the flagellae of *E. coli* O157 may possess adhesive properties. To examine this, flagellae serotypes H7, H11 and H21 from O157, O26, and O113 EHEC strains respectively were purified by differential centrifugation. To confirm purity the flagellaer preparations were separated by SDS-PAGE, analysed by immuno-blotting and colloidal blue staining (Fig. 5.13).

Each of the flagellae preparations gave three bands of approximate molecular sizes for H11 (50, 90 and 110 kDa), H21 (55, 90 and 110 kDa) and a single band for H7 (66kDa). To determine whether these were contaminants or flagellae isoforms samples of each of the 3 main protein bands from each flagellae preparation were analysed by MALDI-MS. All bands in each of the preparations were confirmed as FliC of the appropriate serotype.

The purified flagellae from the different EHEC serotypes were incubated with bovine rectal primary epithelial cells for 3 h and bound flagellae were detected by immunofluorescence (IF) assay using type specific anti-H antibodies after thorough washing to remove loosely associated flagellae. The H7 but not the H11 and H21 flagellae bound to the bovine rectal epithelial cells (Fig. 5.14).

5.3.9 Purified H7 flagellae inhibits *E. coli* O157:H7 binding to Bovine rectal primary epithelial cells

To further test the hypothesis that H7 acts as an adhesin, the bovine rectal primary epithelial cells were pre-incubated with purified H7 flagellae before the addition of bacteria. After pre-treatment of cells for 30 min with purified flagellae, adhesion of *E. coli* O157:H7 was decreased in a dose-dependent manner up to 0.25μg/ml compared with PBS-treated cells ($p \leq 0.042$). Surprisingly, at 4.0μg/ml flagellae significantly enhanced the *E. coli* O157 binding ($p \leq 0.021$) (Fig. 5.15). This pattern was repeated on two replicate assays.

5.3.10 Purified flagellae binds to asialo-GM1

Host cell glycolipids are recognised as initial receptors for certain bacterial flagellae.

For instance, the ganglioside asialo-GM1 has been shown to act as a co-receptor for

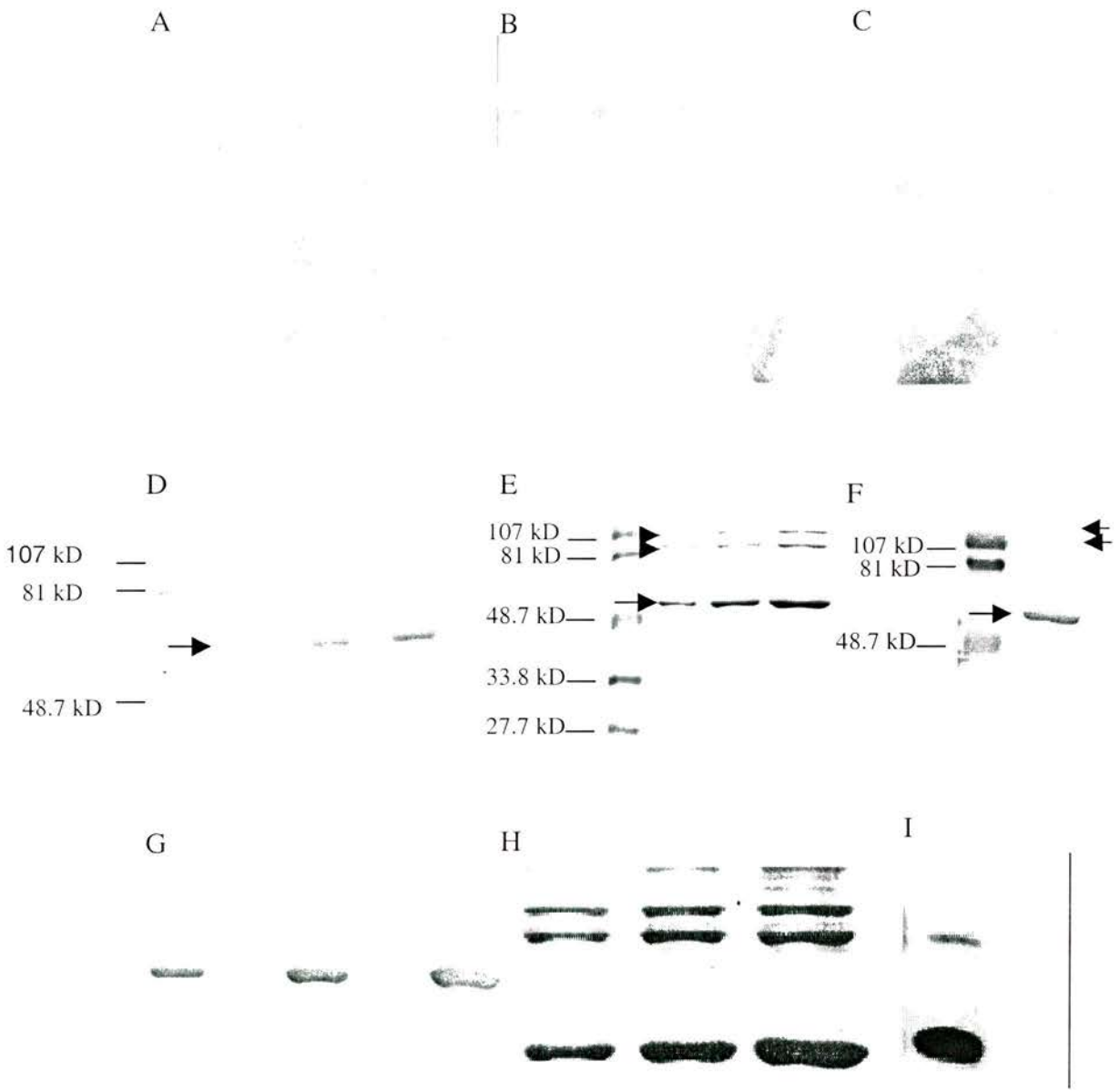

Fig. 5.13 Electron microscopy, SDS-PAGE and immunoblot analysis of purified flagellin proteins from different EHEC strains. Flagella were mechanically sheared and separated by differential centrifugation. Transmission electron micrographs of flagellar filaments from *E. coli* O157:H7 (ZAP 734) (A), *E. coli* O26:H11 (ZAP 116) (B) and *E. coli* 113:H21 (ZAP 244) (C). The isolated flagella were resolved on a 12% polyacrylamide gel and stained with colloidal blue. Major flagellin bands H7 (66kD) (D), H11 (50, 90 and 110 kDa) (E) and H 21 (55, 90 and 110 kDa) (F) are indicated by adjacent arrows. Gels (D&E) and immunoblots (G&H) shows thee lanes of different quantities of the same sample. Flagellar proteins H7 (G), H11 (H) and H21 (I) were transblotted to nitrocellulose membranes, stained with type specific anti-sera, detected with horseradish peroxidase-conjugated secondary antibody.

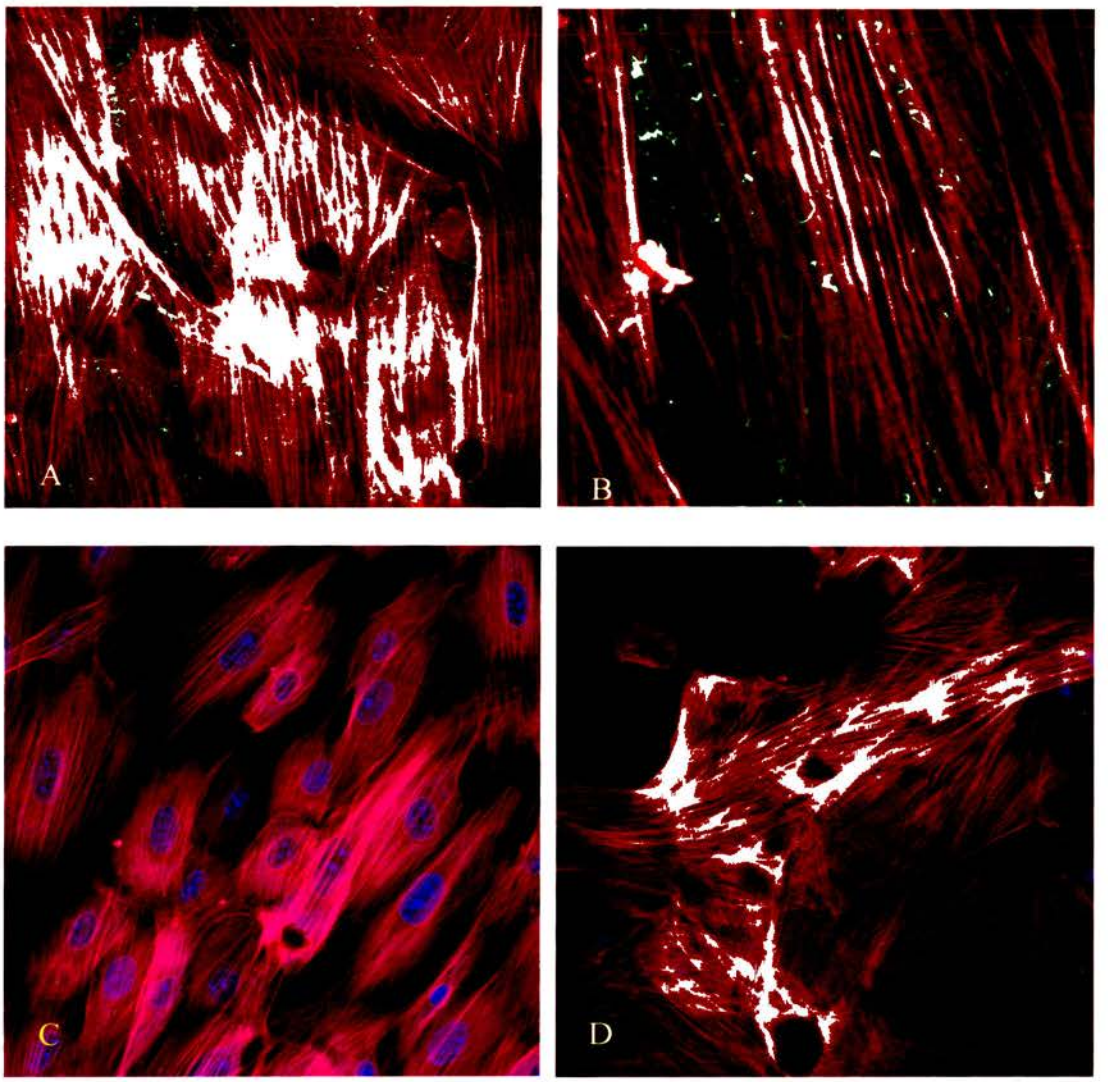

Fig. 5.14 Binding of purified flagella H7, H11 and H21 to bovine rectal primary epithelial cells. The epithelial monolayers were incubated with purified flagella (1μg/ml) from *E. coli* O157:H7 or *E. coli* O113:H21 or *E. coli* O26:H11 at 37°C, 5% CO_2 for 3 h. After thorough washing, the flagella bound to cells were labelled with type- specific anti-H7, anti-H11 and anti-H21 flagellar antibodies and detected with secondary FITC (green) conjugated antibody. Flagellar fragments of H7 (A, B) but not of H11 (C) and H21 (D) are seen adhering to cell monolayer, confirming the adhesive properties of H7 flagella. The epithelial cells have been stained with actin specific phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The images were captured using Leica TCS NT confocal microscope (x 63 objective lens). Image (B) was digitally magnified by a factor of 2.

flagellae from *Pseudomonas aeruginosa* and *Salmonella enteritidis* (McNamara *et al.*, 2001a;Ogushi *et al.*, 2004). Glycolipid binding may be a phenomenon common to a wider range of flagellae therefore purified flagellae from different EHEC serotypes was assessed for binding to asialo-GM1. As shown purified H7 and H11 flagellae bound to the asialo-GM1 in the immuno-blot assay (Fig. 5.16) however binding of H21 flagellae could not be detected. This may be a result of titre of antibody reagents rather than absence of binding since H21 directly dotted onto nitrocellulose membrane was not detected by the relevant antibody. Nevertheless, this finding shows that asialo-GM is one of the potential receptors for EHEC flagellae.

5.3.11 Effect of ganglioside synthesis inhibitor

The bovine rectal epithelial cells were treated with glucosylceramide synthase inhibitor, PPMP, at 50 and 100 μm concentration for 24 h prior to adherence assay with ZAP 734 and ZAP 735. These concentrations were toxic to the cells resulting in their detachment and hence the conditions require to be standardized.

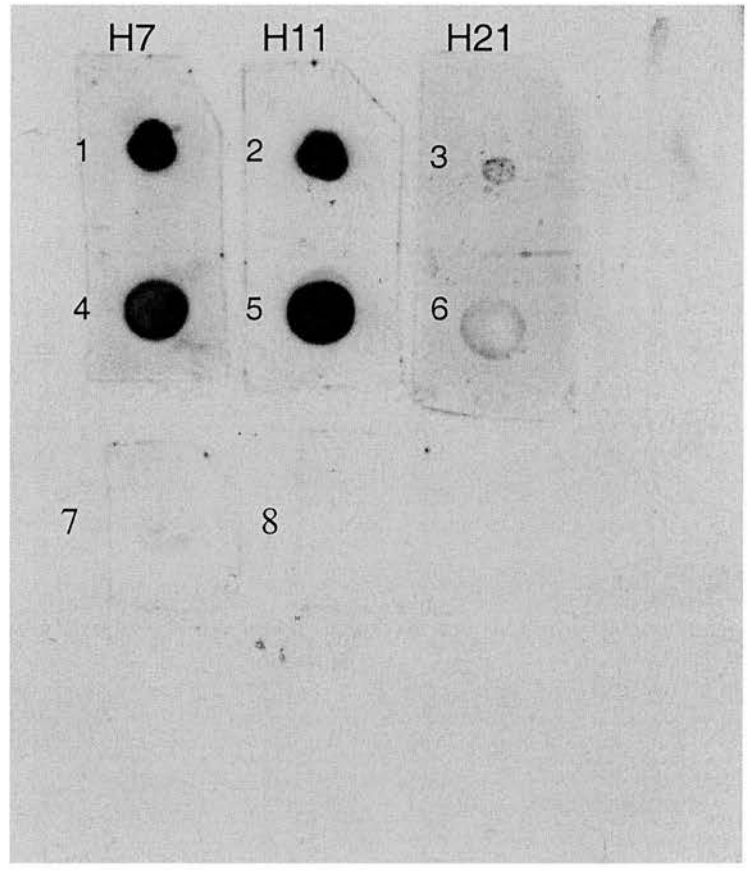

Fig. 5.16 Adherence of purified flagella to asialo-GM1 in Dot-blot binding assay. Aliquots (2μl) of asialo-GM1 (Spot 4-8) and 700μg of each of purified flagellin: H7 (Spot 1), H11 (Spot 2) and H21 (Spot 3), as positive controls, were applied to thee Immobilon-P membranes (Millipore Corp., Bedford, Mass). Specific controls for primary (Spot 7) and secondary (Spot 8) antibodies were employed to test the non-specific binding. Membranes were dried (37°C for 15 minutes) and blocked overnight in 3% BSA–PBS 0.1% Tween-20 solution at 4°C on a shaking platform. The membranes were washed with PBS-0.1%Tween-20 (2 x 30 minutes) and incubated overnight with 700 μg of type specific purified flagellar suspension in PBS-0.1% Tween-20 at 4°C on a shaking platform. The membranes were washed in PBS-0.1% Tween-20 at 4°C (3 x 30 minutes). Adherence of different flagellar types (H7, H11 or H21) to asialo-GM1 was detected with flagellin type specific antisera followed by horseradish peroxidase-conjugated secondary. The membranes were developed in enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Arlington Heights, IL).

5.4 Discussion

Adherence to intestinal epithelium is a key step in colonization and pathogenesis of enteric pathogens including enterohaemorrahagic *E. coli* (EHEC). Adherence is mediated by adhesins on the surface of microbe which bind to specific receptor moieties on host cells and hence define the tissue tropism or host specificity. Microbes possess multiple adhesins that are expressed in a temporally and spatially regulated manner. Expression of adhesins is controlled by various environmental cues or by cross-talk between individual adhesin loci. Therefore colonization is a complex and multiphasic event at the host-pathogen interface.

Strains of EHEC O157:H7, the most common serotype associated with human infections, produce multiple determinants with Shiga toxin as the key virulence factor associated with the disease pathology. *E. coli* O157:H7 possesses an array of adhesins that play a role in intestinal colonization. LEE-encoded type III secretory proteins including EspA, intimin-Tir and other effector proteins play a key role in intimate adherence and the characteristic A/E lesion formation. Numerous other factors including outer membrane proteins (Iha, OmpA) (Tarr *et al.*, 2000;Torres and Kaper, 2003), fimbrial proteins (curli, LPF1, LPF2 and Loc 8) (Kim and Kim, 2004;Torres *et al.*, 2002a;Uhlich, Keen, and Elder, 2002;Jordan *et al.*, 2004;Dziva *et al.*, 2004) ToxB and Efa1 (Nicholls, Grant, and Robins-Browne, 2000;Stevens *et al.*, 2002a) have been identified as *E. coli* O157:H7 adhesins. However, the factors playing a role in the early phase of *E. coli* O157:H7 colonisation are still obscure. In the wake of recent reports on the role of flagellae as an adhesin for EPEC (Giron *et al.*, 2002), a related pathotype to EHEC – the possible role of H7 as an adhesin was investigated.

The adherence data from this study indicates that flagellae are important for the binding of *E. coli* O157:H7 to bovine rectal epithelial cells. A *fliC*-negative mutant of strain NCTC12900 showed significant reduction in adherence to bovine rectal primary epithelial cells ($p \leq 0.0001$). Studies with non-flagellaeted isolates of *C. jejuni* (Morooka, Umeda, and Amako, 1985;Pavlovskis *et al.*, 1991), *H. felis* (Eaton *et al.*, 1996;Eaton, Morgan, and Krakowka, 1992) and *P. aeruginosa* (Feldman *et al.*, 1998b) have demonstrated the requirement of both flagellum and motility for

virulence and colonization. In *P. aeruginosa* for example the flagellum is considered to act in initial interactions and tether the bacterium to the host epithelium (Feldman *et al.*, 1998b). Therefore reduced adherence of *fli*C-mutant strains might be due to loss of motility or chemotaxis, or to the loss of flagellae as an adhesin.

To determine whether motility or the possession of flagellae *per se* were required for adherence of *E. coli* O157:H7 to epithelial cells, the motility defect of the *fliC* mutant was overcome through gentle centrifugation of bacteria onto the epithelial monolayer. This procedure was adopted from previous studies with *Salmonella typhimurium* and *Proteus mirabilis* which demonstrated that the partial recovery of cell association and invasion was achieved by the mild centrifugation of *fli*C mutant bacteria onto the cells (Eichelberg and Galan, 2000;La Ragione *et al.*, 2003;Mobley *et al.*, 1996a;Mobley *et al.*, 1996b). Centrifugation partially recovered the adherence of the *fli*C-mutant strain but the adherence was still significantly reduced compared to the wild-type flagellaete ZAP 734 strain ($p \leq 0.0003$) i.e as expected brief centrifugation increased adherence of both motile and *fliC*-ve strains however the mutant did not attain levels of adherence equivalent to the parent strain. This indicates that flagellae (specifically FliC) directly contribute as an adhesin. Consistent with the findings of others, the role of flagellae is two-fold since motility is important but the possible role as an adhesin is required to recover the complete adherent phenotype of *E. coli* O157:H7. Flagellae are probably acting as an important factor in the initial interaction of *E. coli* O157:H7, at first possibly by enabling the juxtaposition of bacterium and host cell, and secondly acting as an adhesin. After this initial contact other factors are involved in anchoring the bacteria to cells. For instance the LEE components are upregulated by 15 min post-contact with cells (unpublished data) thus EspA translocon, intimin-Tir and other determinants mediate intimate adherence.

Immunofluorescent microscopic investigations revealed abundant expression of H7 flagellae on contact with epithelium during early stages (up to 1 h) of *E. coli* O157:H7 infection, with clear contact points between flagellae and epithelial cells. Structural analysis by SEM of *E. coli* O157:H7 strains adhering to bovine rectal

epithelial cells revealed the presence of flagellae-like structures intercalating with the microvilli. It appeared that H7 might be acting as an adhesin. This role was further indicated during adhesion inhibition assays in which pre-treatment with anti-H7 antibodies significantly reduced the binding of wild type strain *E. coli* O157:H7 but not of the isogenic *fliC* mutant.

This work has shown that H7 flagellae were expressed abundantly by various wild-type *E. coli* O157 strains on adherence to bovine rectal primary epithelial cells and the expression of flagellae varied with the time of infection. At 1 h post-infection, the majority of bacteria expressed flagellae while at 3 h flagellae were rarely expressed by bacteria in the microcolonies. By 8 h post-infection, the adherent bacteria formed typical A/E lesions, characterised by formation of actin-pedestals and bacteria associated with these actin-pedestals appeared to be non-flagellaeted. The formation of actin pedestals is an indicator of functional LEE Type III Secretory Apparatus (TTSS). From these results it appears that an inverse relationship between expression of the TTSS *vis a vis* flagellae may exist in EHEC O157:H7 strains and similar counter regulation systems have been described for *Bordella*, *Salmonella*, and *Yersinia spp.* (Josenhans and Suerbaum, 2002). This counter-regulation between flagellae and TTSS expression may be crucial in EHEC O157:H7 pathogenesis at different stages of infection and a multi-stage process can be proposed: In the early stages of infection, the flagellar apparatus is important for the EHEC chemotaxis towards the target and for the initial adherence. Once EHEC adheres/tethers to the host cell, motility/flagellae is turned off, and other possible adhesins as well as TTSS is activated for more intimate attachment.

The flagellar export apparatus is similar to the bacterial type III protein secretion system (TTSS) (Young, Schmiel, and Miller, 1999;Saier, Jr., 2004;Young, Schmiel, and Miller, 1999). In EPEC and EHEC most of the LEE operons are positively regulated by the Ler regulator, which is encoded by *LEE1* operon (Friedberg *et al.*, 1999;Mellies *et al.*, 1999) (Sperandio *et al.*, 2000;Sanchez-Sanmartin *et al.*, 2001). *LEE1* expression is modulated by various factors that includes Fis, Per and a quorum sensing regulator (Goldberg *et al.*, 2001;Kanamaru *et al.*, 2000;Mellies *et al.*,

1999;Sperandio, Li, and Kaper, 2002;Sperandio *et al.*, 2003;Sperandio, Torres, and Kaper, 2002;Sperandio *et al.*, 2001). An additional regulator, integration host factor (IHF) directly activates the expression of Ler (Friedberg *et al.*, 1999) and thus is required for expression of TTSS. IHF also represses the *flhDC* operon, which encodes the master positive regulator of the flagellaer regulon in EPEC and EHEC (Yona-Nadler *et al.*, 2003). It has been shown that flagellar expression, modulated by IHF, is repressed in culture conditions such as DMEM, which stimulate expression of the TTSS (Yona-Nadler *et al.*, 2003) thus it is plausible to hypothesize similar regulatory processes activating TTSS and suppressing flagellae expression during *E. coli* O157:H7 microcolony and A/E lesion formation.

Down-regulation of flagellae following contact/attachment with epithelium may be important in colonization since flagellae, as one of the pathogen associated molecular patterns (PAMPs), are TLR5 ligands (Hayashi *et al.*, 2001a) act as potent pro-inflammatory molecules. Conserved domains of the major structural component FliC are shared by many different species of bacterial flagellae and function as immuno-stimulatory ligands (Eaves-Pyles *et al.*, 2001b;Eaves-Pyles *et al.*, 2001b). The extracellular domain of TLR5 binds to monomeric flagellin subunits leading to activation of signalling cascades typically converging on the transcription factors NF-κB and AP-1 (Eaves-Pyles *et al.*, 2001a). Flagellin therefore induces expression of a diverse array of innate immune defences, including human β-defensin (Ogushi *et al.*, 2004), mucin (McNamara *et al.*, 2001a), nitric oxide (Eaves-Pyles *et al.*, 2001a), IL-8 (Zhou *et al.*, 2003b) and MIP-3α/ CCL20 (Rhee *et al.*, 2004;Izadpanah *et al.*, 2001). The latter two are important as pro-inflammatory mediators with IL-8 as a PMN chemokine and CCL20 a chemoattractant for CCR6-expressing immature dendritic cells and memory T cells (Sierro *et al.*, 2001;Izadpanah *et al.*, 2001). Studies have shown that *Pseudomonas aeruginosa* lacking flagellae are less inflammatory than flagellaete and are resistant to clearance by macrophages and polymorphonuclear leukocytes (Mahenthiralingam, Campbell, and Speert, 1994;Mahenthiralingam and Speert, 1995). The *fliC* (flagellin) repression in muco-purulent airway liquids collected from chronically infected CF patients may represent an adaptive response that allows *P. aeruginosa* to avoid detection by host

defence mechanisms and phagocytosis during the chronic phase of CF lung infections (Wolfgang *et al.*, 2004) therefore controlling flagellum expression can be important in evading host defences.

Evidently there is a direct association between flagellae and inflammatory responses thus the cessation of flagellae expression at later stages of *E. coli* O157:H7 binding probably represent part of an adaptive response that contributes to the innocuous persistence and colonization of *E. coli* O157:H7 in cattle. Moreover, flagellaer biogenesis and motility are complex processes that involve significant energetic burden to the microbe. For instance, in *E. coli* about 49 genes and 2% of the cell's total energy are required to synthesize and maintain flagellar function (Macnab and Aizawa, 1984). Therefore, it is not surprising that this bacterium strictly controls expression of flagellae since this is both energetically expensive and an immunological liability.

The host-pathogen interaction is an ever-dynamic process with both bacteria and host sensing and responding to each other in communications that can decide the outcome of infection. Bioactive molecules released from host cells have been shown to affect bacterial phenotypes including motility and attachment. For instance, Uhlman and Jones (Uhlman and Jones, 1982) demonstrated that a diffusible chemo-attractant released from HeLa cells enhanced the *Salmonella typhimurium* chemotaxis and attachment. Chaubal and Holt (Chaubal and Holt, 1999) observed that the *in vivo* environment induced motility in *S. enterica* var. *Gallinarum* and *Pullorum* serovars which are traditionally recognised to be non-motile. Similarly in a study by Giron (Giron *et al.*, 2002), it was documented that PCM from HeLa cells activated motility in EPEC strains including the non-motile strains. Recently, Sperandio *et al.*, have shown that both epinephrine and norepinephrine (NE) crosstalk with a bacterial quorum-sensing system regulating LEE expression and motility in *E. coli* O157:H7 (Sperandio *et al.*, 2003). Flagellum synthesis and type III secretion is regulated by an autoinducer (AI-3), the synthesis of which is dependent on LuxS. It is not presently clear whether NE activates LEE expression in *E. coli* O157:H7 by directly substituting for AI-3 synthesis or it stimulates endogenous AI-3 synthesis. NE has

also been shown to augment the adherence of *E. coli* O157:H7 to murine cecal explants *in vitro* (Chen *et al.*, 2003), adherence to intestinal mucosa in a bovine ligated ileal loop model (Vlisidou *et al.*, 2004) and invasion of the porcine jejunal mucosa (Green *et al.*, 2003). During this study the effect of secretory signals from the bovine primary epithelial cells on adherence was examined. The wild type EHEC strain ZAP 734 and the isogenic *fliC*-mutant ZAP 735 were incubated in medium preconditioned by growth of bovine rectal primary epithelial cells (PCM) prior to the adherence assay. The PCM treatment significantly enhanced binding of wild type strain ZAP 734 in comparison to the *fliC* mutant ZAP 735 ($p<0.0006$). It was hypothesised that the increased binding might be related to the expression of flagellae. To test this hypothesis ZAP 734 was incubated in PCM or MEM-HEPES and the expression of flagellae was quantified. The PCM increased H7 expression and thus the enhanced adherence of ZAP 734 following pre-incubation in PCM indeed correlates the effect of flagellaer expression, although the role of other possible factors cannot be ruled out. Although the biochemical characterisation of soluble activator(s) in PCM is underway it is tempting to speculate that a molecule(s) similar to one secreted by bovine primary rectal epithelial cells which trigger(s) expression of flagellae and possibly other virulence factors might be present in the bovine host at the site of *E. coli* O157 colonisation.

One of the hallmark features of ligand-receptor interaction is the ability of the purified ligand to competitively inhibit the bacterial ligand binding to its cognate receptor in dose-dependent manner. The ability of H7 flagellae to block adhesion of *E. coli* O157 to bovine rectal primary epithelial cells was investigated. As shown in Fig. 5.15 purified flagellae inhibited *E. coli* O157:H7 binding to bovine rectal primary epithelial cells in a dose-dependent manner upto 0.25μg/ml. At flagellae concentrations >0.25μg/ml, the inhibitory effect began to diminish, and surprisingly, 4.0μg/ml flagellae enhanced *E. coli* O157:H7 binding. Interestingly, a similar enhancement of adherence of *Salmonella enteritidis* to ovarian granulosa cells (M.M.Popielarczyk1, 1999) and *Pseudomonas aeruginosa* to CHO-Muc1 cells (Lillehoj, Kim, and Kim, 2002) by purified flagellae have been reported. The initial reduction in binding of *E. coli* O157:H7 on treatment with purified flagellae at

0.25μg/ml concentration might be that flagellae saturates its potential receptor on the apical surface. The mechanism(s) by which high dosage enhances the adherence remains unexplored but it is possible that at higher concentration flagellae activates either through TLR5 or unknown mechanism(s), expression of novel receptor(s) or secretory molecule(s) that enhance(s) the bacterial-cellular interaction or may increase the bactera-bacteria interactions.

Isolated H7 flagellae bound to bovine rectal primary epithelial cells confirming that these flagellae *per se* are adhesive structures. It was observed that H7 flagellae showed higher binding than H11 and H21 to bovine rectal primary epithelial cells. This work does not explain the mechanism of binding of flagellae but it is possible that H7 flagellae possesses certain unique binding domains that confer its preferential binding to certain motifs and thus differential cellular tropism. Flagellin, the monomeric subunit of flagellae is composed of conserved domains (D1 and D2) at the N- and C-terminal regions with a central hyper-variable domain (D3). The conserved residues in the D1 domain are required for recognition via TLR5, however these regions are "cryptic" or obscure in intact flagellae (Eaves-Pyles *et al.*, 2001b;Smith *et al.*, 2003a). The non-conserved D3 domain is exposed on the surface of flagellaer filament and contains the major antigenic epitopes that confer the sero-specificity and possible putative adhesion domain(s) (McDermott *et al.*, 2000;Steiner *et al.*, 2000). Comparison of FliC sequences of H7, H11 and H21 confirms that the distinct motifs in the central region of FliC might confer differential ligand and cellular tropism. Since the TLR5 binding domain of flagellin is not exposed (Eaves-Pyles *et al.*, 2001b) and as TLR5 is usually located basolaterally in enterocytes (Gewirtz *et al.*, 2001b;Gewirtz *et al.*, 2001a) it is unlikely that TLR5 is an initial receptor for flagellin. Therefore the working hypothesis is H7 flagellae initially binds via putative central adhesin domain, to an unknown receptor on the apical surface conferring attachment and later monomeric flagellin interact with the TLR5 to initiate pro-inflammatory responses.

Other epithelial receptors or co-receptors for flagellae have been proposed, including TLR2 (Adamo *et al.*, 2004), gangliosides (McNamara *et al.*, 2001b;Ogushi *et al.*,

2004) and mucin (Lillehoj, Kim, and Kim, 2002) therefore several complementary means of flagellae-epithelium interaction may operate. Gangliosides, sialic acid–containing glycosphingolipids, are ubiquitous components of eukaryotic cell membranes that have been identified as receptors for bacterial toxins and viruses (Orlandi and Fishman, 1998;Fishman, Pacuszka, and Orlandi, 1993;Fishman, 1982;Markwell, Svennerholm, and Paulson, 1981). Although TLR5 has been determined as the flagellin receptor, gangliosides such as GM1, GD1a, and asialo-GM1 were also shown to bind to *Pseudomonas aeruginosa* flagellin in an *in vitro* binding assay (Feldman *et al.*, 1998a). Asialo-GM1 mediated activation of phospholipase C, Ca^{2++} mobilization and phosphorylation of ERK1/2 was found critical in stimulating transcription of the mucin *Muc 2* (McNamara *et al.*, 2001b). Recently, Ogushi *et al.,* (Ogushi *et al.*, 2004) reported that gangliosides act as co-receptors with TLR5 for *Salmonella enteritidis* flagellin and promoted hBD-2 expression via MAP kinase. Yu *et al.*, (Yu *et al.*, 2003) reported that induction of p38 MAP kinase phosphorylation by *Salmonella typhimurium* flagellin was mediated by activation of TLR5 rather than through asialo-GM1. These glycolipids may therefore be important not only in flagellae binding but also in mediating host cell responses to bacteria.

To investigate asialo-GM1 as a possible ligand to EHEC flagellae, immuno-blot assays were conducted. Binding of H7 and H11 flagellae to purified asialo-GM1 identified it to be a possible receptor(s) and work is in progress to examine the role of gangliosides as possible ligands to EHEC flagellin on bovine gut epithelium. The bovine rectal epithelial cells were treated with ganglioside synthesis inhibitor PPMP at 50, and 100 μm concentration for 24 h prior to adherence assay with ZAP 734 and ZAP 735. These concentrations were toxic to the cells and hence protocols require further refinement. The working hypothesis is that *E. coli* O157:H7 might use flagellum to "browse" the epithelial surface, and then to tether the organism to an exposed site with the accessible GM1 or asialo-GM1 as a very early event in establishing a nidus of infection. Glycolipid receptors such as ASGM1, GM1 & Gb3 can be present in "rafts" or "microdomains" often associated with trans-membrane signalling molecules thus forming receptor-signalling complexes. Engagement of

glycolipids by ligands like flagellin can thus affect cellular physiology leading to multiple host responses that may make epithelial membrane permissive to binding (Adamo *et al.*, 2004;McNamara *et al.*, 2001b). This might be one of the mechanisms whereby pre-treatment with flagellae at a higher concentration enhanced the binding of *E. coli* O157:H7 to bovine rectal epithelial cells

This work has shown that H7 flagellae facilitate attachment of *E. coli* O157:H7 to bovine intestinal (rectal) epithelial cells. H7 acts an adhesin, and plays role in initial interaction of EHEC serotype O157 with epithelial membranes. These findings differ in detail from those of Giron et al. (Giron *et al.*, 2002) who demonstrated that H7 flagellae were not expressed during contact with human epithelial cells (at least at those time points they examined) although that investigation employed HeLa and Hep-2 cells (neither of which are intestinal in origin). The role of flagellae in adherence of *E. coli* O157:H7 was found to be stage dependent. At early stages of interaction the individual adherent bacteria abundantly expressed flagellae whilst at later stages the bacteria attached via actin pedestals (A/E lesions) or microcolonies were non-flagellaeted. Down-regulation of flagellae, a potent proinflammatory stimulus of EHEC and other enteropathogens, at later stages of adherence is probably a microbial mechanism adapted to attenuate the proinflammatory responses and facilitate *E. coli* O157:H7 colonization. The flagellated wild type strain formed compact microcolonies as compared to the sparsely adherent non-flagellaete isogenic mutant strain, indicating the role of H7 in microcolony formation.

On contact with bovine rectal epithelial cells, *E. coli* O157:H7 strain expressed flagellae and formed compact microcolonies where as other EHEC O26:H11 and O113:H21subtypes adhered sparsely and did not express flagellae. Although more experiments are required to examine if the differential expression of flagellae by EHEC subtypes is affected by specific cell type used in this study. It is tempting to speculate that expression of flagellae by O157 might determine the unique tropism of this serotype to bovine terminal rectum.

Many receptors for adhesins, including flagellae, are glycoconjugates (glycoproteins or glycolipids) hence the future work aims at assessing the binding of *E. coli* O157:H7 and isolated flagellae (H7 and others) to epithelial cells both before and after deglycosylation with glycosidases or periodate. Inhibitors of protein glycosylation and glycolipid synthesis are also being employed in preliminary characterisation of putative receptor(s).

However the role of H7 flagellae as an adhesin in cattle *in vivo* still remains to be eastablished. As part of future work, calf challenge experiments will be done with flagellated and isogenic non-flagellated *E. coli* O157:H7 strains to compare the extent of colonisation.

6. Conclusions

E. coli O157:H7 exhibits a novel tropism for the terminal rectum in the bovine host. In experimentally-infected and naturally-colonised animals the majority of tissue-associated bacteria have been identified in a region within five centimetres proximal to the recto-anal junction (Naylor *et al.*, 2003). To aid in defining determinants involved in confirming this tropism this work aimed to:

1. characterise epithelium and associated lymphoid follicles for features which may determine the bovine terminal rectal tissue-specific tropism of *E. coli* O157:H7 in cattle (Chapter 2).
2. develop and characterise primary epithelial cell culture from terminal rectum as an *in vitro* model to examine EHEC interaction with bovine intestinal epithelium (Chapter 3).
3. determine roles of bacterial verotoxin and flagellae on interaction with epithelium (Chapter 4 and Chapter 5 respectively).

The mucosa at this site contrasts with the majority of the large intestine in possessing a high density of lymphoid follicles in dense patches in the region 0-3 cm proximal to recto-anal junction. This localisation of LFs may be responsible for the tropism shown by *E. coli* O157:H7 for this site. Immunohistological investigations showed that rectal FAE, like that of PP, contains many M-cells. These cells ingest macromolecular antigens and pathogens, therefore *E. coli* O157:H7 may preferentially bind to M-cells although this remains to be demonstrated (see chapter 3). The FAE/M-cells are typically marked by the presence of distinct glycoconjugates that are potential ligands for many microbial adhesins; however using lectins this study could not define saccharide residues specific to FAE or M-cells in the bovine terminal rectum. It will be crucial to define and compare to lymphoid-follicle-rich tissue at other sites, to have a better understanding of the host determinants of *E. coli* O157:H7 interaction at this site.

Immunophenotyping of bovine terminal rectal mucosa indicates that lymphoid follicles contain antigen-presenting cells, T- and B- lymphocytes and is therefore likely to be an immunologically active site. Consequently, the tropism of *E. coli* O157:H7 to this site could be hazardous for the bacterium and therefore *E. coli* O157:H7 may need to restrict its uptake or otherwise interfere in host defences. Tropism of *E. coli* O157:H7 for this short lymphoid-follicle dense site in the most terminal region of GIT might have evolved to provide minimal contact with the host tissue and to effectively limit the immune response whilst maintaining persistent shedding in the faeces at relatively high levels. This localisation could limit the induction of both innate immune responses that could lead to clearance of the organism and restrict development of adaptive immune responses that would limit subsequent colonisation. Bacterial factors could then further down-regulate such immune responses by exported bacterial factors and hence lead to innocuous persistence and colonisation of *E. coli* O157:H7 in the bovine host.

Intestinal epithelium is comprised of multiple cell types, including absorptive, secretory (goblet), enteroendocrine, Paneth and M-cells that arise from pluripotent stem cells present in intestinal crypts. Primary epithelial cell cultures were developed to provide an *in vitro* model to examine EHEC interaction with the bovine host. Primary epithelial culture, having mixed population of cells including proliferating crypt-like cells, differentiated epithelial cells and goblet cells, closely resembled a native intestinal epithelium. A subset of cells in the culture expressed vimentin and trancytosed microparticles thus phenotypically and functionally resembled antigen sampling M-like cells *in vivo.* This system provides a relevant *in vitro* model to examine interaction of EHEC with the bovine host.

It may be anticipated that *E. coli* O157:H7 interaction with FAE occurred as a result of M-cell sampling activities however in this study *E. coli* O157:H7 diffusely adhered to epithelial cells with no preference to FAE/M-like cells expressing vimentin or cells taking up microparticles. The adherence of these bacteria to both M-cells and enterocytes suggests other determinants e.g presence of receptor(s) specific to, or expressed preferentially, at this site. The significance of this

observation needs to be established following quantitative adherence assays. *E. coli* O157:H7 tropism for bovine rectal FAE, and its molecular basis are active areas of investigation.

Verotoxins (VTs) are potent cytotoxins that are closely associated with severe pathological consequences of EHEC infections in humans. Although the toxic and proinflammatory activities of VTs have been demonstrated extensively *in vitro* and *in vivo*, *E. coli* O157 produces no overt disease in cattle, its primary reservoir host. The presence of VT in faeces of cattle (Ball *et al.*, 1994;Hyatt, Galland, and Gillespie, 2001) and the detection of anti-VT antibodies in bovine sera and colostrum (Johnson, Cray, Jr., and Johnson, 1996;Pirro *et al.*, 1995) provides evidence that VT is produced in cattle but has no demonstrated role in local and systemic cytotoxicity or pro-inflammatory activity. Therefore, it was hypothesised that VTs may be able to modulate intestinal inflammation in the bovine mucosa.

This work showed that in cattle VT induced IL-8 synthesis and secretion thereby contributing to limiting local inflammatory responses. Surprisingly, VT also enhanced adherence of VTEC (*E.coli* O157:H7 and others) to bovine rectal epithelial cells. It is possible that VTs could alter the host cell surface, thereby providing the EHEC with alternate novel receptors or enhancing expression of constitutive receptors. It is a part of ongoing work in the lab to understand the molecular basis to VT dependent IL-8 suppression and enhanced binding of EHEC to bovine rectal epithelial cells.

A necessary step in the successful colonization and, ultimately, production of disease by microbial pathogens is the ability to adhere to host surfaces. *E. coli* O157:H7 factors conferring initial interaction to intestinal epithelium have remained obscure. In this study, H7 flagellae were identified as adhesins and a contributory factor for microcolony formation. The *E. coli* O157:H7 interaction with epithelium is complex and multiphasic, and is likely to involve multiple ligand-receptor contacts during course of colonization. The role of H7 flagellae as an adhesin was apparent only during early stages of interaction as, at later stages, bacteria associated with actin-

pedestals or in microcolonies did not express flagellae. The cessation of flagellar expression at later stages of infection may be a part of microbial adaptive response to circumvent host recognition. The expression of flagellae during adherence to bovine rectal epithelial cells was discernible only with *E. coli* O157 strains but not with serotypes O113 or O26 thus raising important questions: is the expression of flagellae unique to O157 serotype and is it a cell-type specific phenomenon? It will be important to use a larger panel of strains of different serotypes to examine adherence and expression of flagellae in time course experiments on primary epithelial cells from mid- and terminal-rectum.

Summary of EHEC *E. coli* O157:H7 **interaction with bovine rectal epithelium.**

The above findings supplement our understanding of *E. coli* O157:H7 colonisation, particularly of the cattle reservoir. A model for this can be summarised as follows: During host-pathogen interaction, motility can be important in facilitating successful colonisation. Adherence to epithelium is an early and essential step in colonisation by enteropathogens, including EHEC in cattle. Adherence is a complex and multi-phasic event that involves strict regulation of various adhesin-receptor interactions. The adhesin/receptor expression during EHEC interaction with intestinal epithelium is temporally and spatially controlled by various physiological and environmental cues.

Flagellae are important as locomotory organelles for the *E. coli* O157:H7 chemotaxis to the targeted site. Epithelial signals up-regulate expression of H7 flagellae which are presumably important for promoting chemotaxis towards potential target cells for initial interaction with epithelial cells. H7 flagellae also act as adhesins during initial stages of *E. coli* O157:H7 interactions with the epithelial membranes. Once *E. coli* O157:H7 tethers to the host cell via flagellae, intimate attachment characterised by formation of actin pedestals and compact microcolonies (TTSS- dependent process), occurs. During this process, H7 flagellar expression is turned off because of it being energetically expensive and potent proinflammatory stimuli.

Verotoxin, a secreted bacterial factor, also has a role during early adherence events since it promotes attachment perhaps via inducing expression of epithelial receptor(s). Adherence of pathogen to host cells evokes modulators of immune response which serve to signal and recruit phagocytic cells to the site of infection. IL-8 is one such potent chemoattractant and activator for neutrophils, an early line of immune defence. Microbes have acquired various tactics to evade host defence mechanisms including suppression of inflammatory cytokines. Down regulation of H7 flagellae will aid evasion of inflammatory responses initiated via epithelial TLR5, the flagellin receptor. Although important in initial attachment, LEE-encoded factors and perhaps fimbriae and non-fimbriated adhesions will continue to confer adherence in absence of flagellae. VT is another EHEC factor involved in evading inflammation. VT suppressed IL-8 synthesis/ secretion and thus may attenuate local mucosal immune responses in the bovine host. In conclusion, H7 flagellae and VTs can be added to the determinants of *E. coli* O157:H7 involved in colonisation and persistence at the bovine rectal epithelium.

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Appendix I

Cell and Tissue Research

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Regular Article

Phenotypic and functional characterisation of follicle-associated epithelium of rectal lymphoid tissue

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Abstract Lymphoid follicles cluster in the terminal rectum of various animal species and of man and hence this site may be important in the development of immune responses to pathogens. For the induction of immune responses at mucosal sites, interplay is required between various cell types performing functions ranging from antigen-sampling cells via antigen-presenting cells to antigen-specific lymphocytes. Therefore, we have characterised the cell populations and relevant functioning of follicle-associated epithelium (FAE) and associated follicles in the terminal portion of rectum in cattle as a representative mammal. Immunohistochemical studies of this region identified immune cell subsets (CD4+, CD8+, WC1+ $\gamma\delta$, CD2+, CD21+ and CD40+ cells) characteristic of an immune-inductive site.

Examination of FAE identified a subset of cells with structural and functional features of antigen-sampling M-cells. Cells of the FAE and adjacent follicle-associated crypts expressed vimentin and a subset of these cells internalised microparticles, a further attribute of M-cells. The FAE cells were phenotypically heterogeneous and therefore the function and phenotype of these cell subsets requires further characterisation, particularly with respect to their potentially important role in the interaction of hosts with pathogens and the development of immune responses.

Keywords Lymphoid follicle - Follicle-associated epithelium - M-cell - Microparticle

uptake - Bovine

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Introduction

The mammalian intestinal surface is exposed to a multiplicity of components within the luminal contents. Among important functions of the epithelium is the prevention of infection by potentially pathogenic micro-organisms. Factors, such as the brush-border glycocalyx (Frey et al. *1996*), are involved in limiting the access of microbes, their components or other potentially detrimental factors to the epithelial surface, whereas intraepithelial tight junctions (Madara et al. *1990*) prevent their access to sub-epithelial tissues. Other factors secreted by the epithelium are also important in the protection of mucosal surfaces, including mucins, antimicrobial peptides and antimicrobial enzymes (Mashimo et al. *1996*; Ouellette and Selsted *1996*). To complement innate defences, antigen-specific defences including immunoglobin secreted via the epithelium are also activated and, hence, the adaptive immune system must sample antigens in order to mount antimicrobial protective responses. Mechanisms have evolved whereby particulate material and macromolecules can be transported across intact mucosal surfaces for presentation to lymphoid tissue. This function is performed principally by M-cells (membranous or microfold cells; Owen and Jones *1974*) but also by other cells, such as the dendritic cells (Rescigno et al. *2001*) of the reticulo-endothelial system that are typically situated in the epithelium overlying organised foci of lymphocytes known as lymphoid follicles (LF).

The organisation and composition of intestinal LFs (collectively termed gut-associated lymphoid tissue; GALT) varies between the different regions of the intestine within species and also between species. Gut LF may be distributed as discrete individual follicles or may form aggregates at anatomically defined sites, e.g. the Peyer's patches of the terminal ileum (Nagi and Babiuk *1988*, *1989*). The epithelial cells overlying LFs are distinct from other epithelia and contain M-cells as a significant component. Reflecting the heterogeneity in GALT, the follicle-associated epithelium (FAE) overlying LFs exhibits heterogeneity with respect to features including its general morphology, its proportion of M-cells, the ultrastructure of the M-cells and the complement of surface receptors (Gebert et al. *1992*; Jepson et al. *1992*). For example, in comparison with gut enterocytes, FAE and, in particular, M-cells express a reduced glycocalyx (Frey et al. *1996*; Helander et al. *2003*) and readily take up foreign particles and micro-organisms.

Several micro-organisms exploit FAE or M-cells for attachment or invasion. For instance, certain viruses (including HIV-1, reovirus type-1 and poliovirus), enteroinvasive bacteria (including *Shigella flexneri*, *Salmonella enterica* serovars Typhi and Typhimurium, *Yersinia enterocolitica* and *Y. pseudotuberculosis*) and extracellular bacterial pathogens (including *Vibrio cholerae*, *Campylobacter jejuni* and *Escherichia coli* RDEC-1) target FAE/M-cells (Kraehenbuhl and Neutra *2000*) during infection. Recent reports that verotoxigenic *E. coli* O157:H7 localises to FAE of the terminal rectum of cattle, its main reservoir host, within a small region defined by the presence of lymphoid aggregates (Naylor et al. *2003*; Rice et al. *2003*) is consistent with the tropism for FAE displayed by other enteric bacteria. Therefore, FAE and, in particular M-cells, are crucial interfaces between the intestine and micro-

organisms.

In view of the importance of M-cells and FAE in this interaction between host and pathogens, we have characterised the lymphoid tissue and associated epithelium of the bovine terminal rectum. The bovine system forms the basis for the current investigation because reported similarities among mammalian (including human, bovine and ovine; Gebbers et al. *1992*; Langman and Rowland *1992*; Sedgmen et al. *2002*) rectal FAE and LF indicate this to be a representative system in which to study lymphoid tissue and associated epithelium. Specifically, the investigation includes a characterisation of the general morphology, the ultrastructure, the lymphoreticular cell populations within the LFs and the function of M-cells in order to improve our understanding of the biology and role of this site.

Materials and methods

Tissue sampling and gross evaluation of LF distribution

Tissues for all procedures were obtained either from the local abattoir or from animals being used for other experimental purposes and were transported to the laboratory in ice-cold Hanks' balanced salt solution (GIBCO BRL). For tissue obtained from the abattoir, typically 30–45 min elapsed between tissue excision and the initiation of the experimental protocols, whereas for tissue obtained from experimental animals, this period was typically 15 min. Our previous investigations had shown that gastrointestinal tissue integrity and function was retained for at least 3 h after removal from the body (Jackson et al. *2004*). All procedures with animals were subject to ethical review and were performed with appropriate licencing from the Home Office and in accordance with the Animal (Scientific Procedures) Act of the UK.

To assess the distribution of LFs macroscopically in the bovine terminal rectum, tissue was treated with acetic acid (adapted from Cornes *1965*; Chauhan *1970*). The terminal 30 cm of the rectum and anal canal were obtained from five mature cattle at a local abattoir. In the laboratory, the specimens were opened longitudinally and the entire mucosal surface was stripped by dissection from the submucosa and immersed in 70% acetic acid (v/v) overnight. LFs became visible as white nodules on the submucosal surface. The distribution of LFs was the same in all animals examined; representative results are presented.

Processing for histology

The terminal 30 cm of the rectum was obtained at necropsy from four male, clinically healthy, Holstein-Friesian calves aged between 8 and 12 weeks. For immunohistochemistry, full-thickness tissue from the regions 0–2 cm and 18–20 cm proximal to the recto-anal junction (RAJ) was fixed in zinc acetate fixative (ZSF; Gonzalez et al. *2001*) consisting of 0.1 M TRIS-base buffer, calcium acetate (0.05% w/v), zinc acetate (0.5%w/v) and zinc chloride (0.5% w/v), pH 7.4. After 6–8 h of fixation, the tissue samples were trimmed to 3–4 mm thickness and further fixed overnight in ZSF at room temperature. The fixed tissue samples were transferred to 70% ethanol (v/v) before being processed to paraffin wax. Sections (5 μm) were cut, mounted on coated slides (Superfrost Plus, Menzel Glaser, Germany) and dried overnight at 37°C. For cryosectioning, tissue samples were immersed in

Tissue-TEK optimum cutting temperature embedding medium (OCT; BDH, Poole, UK), snap frozen in liquid nitrogen and then stored at –20°C. Frozen sections were cut at 8–10 μm (Shandon Cryostat, UK) and mounted on Superfrost microscope slides. The cryosections were air-dried, fixed in methanol cooled to –20°C and subsequently rehydrated for 10 min in phosphate-buffered saline (PBS) prior to staining.

Immunohistochemistry

Sections from paraffin-embedded tissue were de-waxed and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 min. After treatment with 25% v/v normal goat serum (Vector Laboratories, Burlingame, USA) for 30 min, sections were incubated (overnight at 4°C) in a panel of monoclonal antibodies specific to various cell markers (Table 1). Primary antibody was detected by using goat anti-mouse horseradish peroxidase (HRP)-labelled antibody (Horizontal En Vision Plus HRP system, Dako, Ely, UK; 30 min incubation at room temperature). The sections were developed in a citrate buffer containing 3,3′diaminobenzidine hydrochloride (DAB; Vector Laboratories) for 7–8 min, counterstained with haematoxylin and mounted in DPX (BDH Laboratory Supplies Poole, UK). TRIS-buffered saline was used to wash tissue sections between each stage of the labelling procedure and as a diluent for the antibodies.

Table 1 Monoclonal antibodies used in immunohistological staining (*IAH* Institute for Animal Health, Compton, UK, *ILRAD* International Laboratory for Research on Animal Diseases or ILRI, Nairobi, Kenya)

Monoclonal antibody	Specificity	Cellular expression	Source	Reference
CC21	CD21	Follicular dendritic cells, mature B cells	IAH	(Naessens and Howard *1991*)
CC20	Bovine CD1b	Dendritic cells	IAH	(Howard et al. *1993*)
CC15	Bovine WC1	$\gamma\,\delta$ T cell	IAH	(Howard et al. *1989*)
ILA-12	CD4	T helper cells	ILRAD	(Teale et al. *1987*)
ILA-51	CD8	Cytotoxic T cells	ILRAD	(Teale et al. *1987*)
ILA-156	CD40	B cells, antigen-presenting cells	ILRAD	(Norimatsu et al. *2003*)
ILA-111	CD25	Activated T and B cells, macrophages	ILRAD	
ILA-30	Ig M	IgM and plasma cells	ILRAD	(Levkut et al. *1995*)
K84 2F9	Ig A	IgA and plasma cells	Serotec	
ILA-43	CD2	$\alpha\,\beta$T cells, natural killer cells	ILRAD	(Gebert *1997*)
ILA-24	MHC-II	Dendritic cells, B cells, macrophages, other antigen-	ILRAD	

		presenting cells		
Anti-vimentin	Vimentin	Cells of mesenchymal origin, M cells	Sigma-Aldrich	(Gebert et al. *1992*)

For lectin-binding studies, a panel of seven biotinylated lectins (Vector Laboratories; Table 2) were used on frozen sections, ZSF-fixed or paraformaldehyde-fixed sections. After the blocking of endogenous peroxidase activity and non-specific binding (as described above), sections were incubated with biotinylated lectins (1 h at room temperature). The sections were developed with streptavidin–HRP conjugate and DAB and counterstained with haematoxylin as above. Sections were washed in PBS (3×5 min) between each step. Sections from mouse Peyer's patch were used as a control to confirm binding by *Ulex europaeus* lectin (UEA).

Table 2 Lectins used in this study (*NALT* nasal-associated lymphoid tissue, *ND* not determined)

Lectin (source)	Carbohydrate specificity	Species/site specificity
UEA-1 (*Ulex europaeus*)	α-L-fucose	M cells in caecum (Gebert and Hach *1993*) and palatine tonsils (Gebert *1997*) of rabbit; Mouse Peyer's patch M cells (Clark et al. *1993*)
WGA (*Triticum vulgaris*)	D-*N*-acetyl glucosamine	M-cells in caecum of rabbit (Gebert and Hach *1993*) and chicken (Jeurissen et al. *1999*); NALT of rat (Takata et al. *2000*)
DBA (*Dolichos biflorus*)	α-D-galNAc	Rabbit caecal M cells (Gebert and Hach *1993*)
PNA (*Arachis hypogaea*)	D-galactose	ND
RCA (*Ricinus communis*)	β-D-galactose	ND
SBA (*Glycine max*)	D-*N*-acetyl galactosamine	M cells in caecum of rabbit (Gebert and Hach *1993*) and chicken (Jeurissen et al. *1999*)
ConA (*Canavalia ensiformis*)	α-D-mannose	ND

The histological characteristics and lectin and immunohistochemical staining of rectal tissues were consistent between animals. Representative results are therefore provided.

Electron microscopy

Samples of tissue from 0–2 cm and 18–20 cm proximal to the RAJ were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). For transmission electron microscopy (TEM), the specimens were post-fixed in a mixture of 1% osmium tetroxide (w/v) and 1.5% potassium ferricyanide (w/v), dehydrated in a graded series of alcohols and embedded in Epon 812 (Fluka). Thick sections (1 μm) were stained with 1% toluidine blue

to identify regions containing FAE, from which ultrathin sections (70–90 nm) were cut. The sections were stained with uranyl acetate and lead citrate and examined in a Philips CM12

transmission electron microscope. For scanning electron microscopy (SEM), fixed tissue was dehydrated in graded acetones, critical-point-dried, sputter-coated with gold/palladium and viewed in a Hitachi 4700 FEG SEM (field emission gun) scanning electron microscope. Characteristics were consistent in tissues obtained from separate animals. Representative results are provided.

Microparticle uptake in vitro and in vivo

Tissue specimens obtained from adult cattle at a local abattoir were transported in ice-cold Hanks' balanced salt solution. The terminal rectal mucosa 3 cm proximal to the RAJ was carefully excised and washed in cold PBS, cut into 1-cm squares with a thickness of 2 mm and placed in RPMI 1640 tissue culture medium (Sigma-Aldrich). The mucosal pieces were placed on a sterile foam pad immersed in prewarmed (37°C) RPMI 1640. An agarose (3% w/v in PBS) collar was placed around the explant with molten agarose being used to seal any gaps between the collar and edges of the tissue.

Aliquots (100 μl) of 1:100 suspensions of latex microspheres of 0.2 μm or 0.5 μm in size (Polysciences, Germany) were pipetted evenly onto the mucosal explants, which were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 45 min. The explants were washed thoroughly three times in PBS and were then placed in OCT and processed for cryosectioning as described above. The cryosections were air-dried overnight, fixed and permeabilised in 2% paraformaldehyde/Triton X-100 (0.25% v/v) for 15 min at room temperature. After being washed with PBS, the sections were stained with phalloidin conjugated to tetramethylrhodamine isothiocyanate (TRITC; 5 μg/ml w/v; Sigma) for 20 min in the dark. Sections from several animals were examined by confocal microscopy. Representative images are presented.

To assess bead uptake in vivo, a 500-μl suspension of 0.5-μm latex microspheres diluted as above in PBS was delivered intra-rectally to clinically healthy, Holstein–Friesian calves aged between 8 and 12 weeks. Tissue was collected from these calves at 20-min intervals from 30 min post-challenge. The calves were killed by an intravenous injection of pentobarbital. The abdomen was opened and the terminal 10 cm of rectum and anus were removed as a single piece. After the rectum had been double-ligated and transected, the anus was circumsected and the pubic bone was reflected. Tissue sections from 0 to 2 cm proximal to the RAJ were fixed in ZSF and processed for cryosectioning as described above.

Confocal microscopy

A Leica TCS NT confocal system was used to acquire a series of optical sections. When required, images were visualised in the Surpass module of the Imaris software suite (Bitplane, Zurich), which converts the image voxels into a geometric object. Following threshold segmentation, the image was then surface-rendered allowing volumes and interactions between objects to be defined.

Results

Distribution and morphology of LFs in the bovine terminal rectum

The majority of the rectal mucosa formed longitudinal and transverse folds and ridges. The mucosa became smoother within a region 4–5 cm cranial to the RAJ (defined by the junction between the columnar and stratified squamous epithelium) and longitudinal folds termed the ampulla recti were present. In this region, high densities of LFs formed confluent patches with solitary follicles becoming more scarce cranially. LFs were rare at more than 5 cm cranial to the RAJ. There was no association between the lymphoid patches and the longitudinal folds at this site.

The LFs at the terminal rectum were observed in two morphologically distinct forms: those showing a single germinal centre capped by a dome layer that did not extend above the level of the surrounding mucosa (Fig. 1b) and those showing several contiguous follicles underlying a pit or diverticulum formed by an inversion of the mucosal surface (Fig. 1c). Protruding dome-like structures lined by FAE were frequently present within these pits (Fig. 1d).

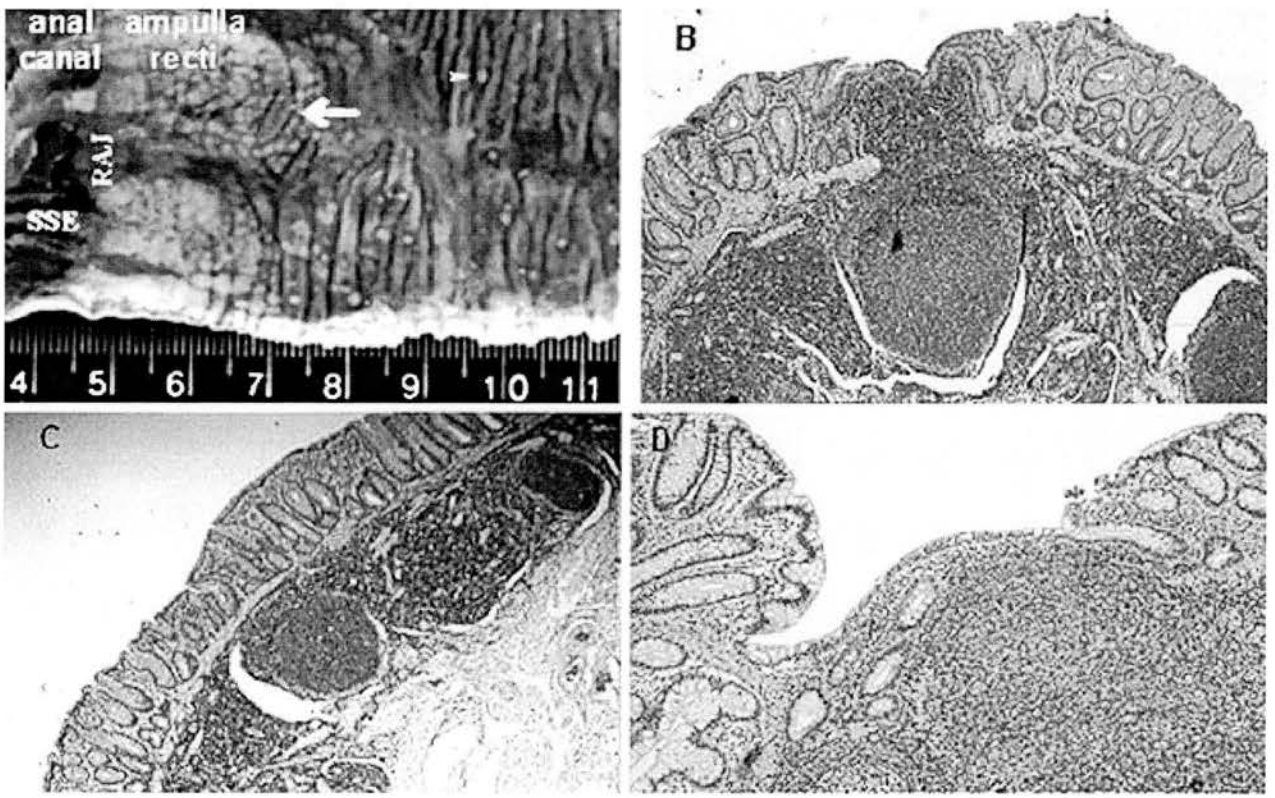

Fig. 1 **a** Terminal portion of rectal mucosa after treatment with 70% (v/v) acetic acid and removal of serosal connective tissue. The anal canal and recto-anal junction (*RAJ*) lie distal to the ampulla recti. Aggregates of lymphoid follicles (LF; *arrow*) are observed at high density in the region 0–3 cm proximal to the RAJ and individual follicles (*arrowhead*) are visible as discrete white nodules (*SSE* stratified squamous epithelium). *Scale* given *below*. **b–d** Haematoxylin and eosin (H&E) stained sections of bovine rectal LFs. **b** An LF in the submucosa extending through the muscularis mucosae into the lamina propria. ×25. **c** Contiguous LFs completely located under the muscularis mucosae. ×25. These were previously referred to as the propria nodule and lymphoglandular complex, respectively (Giannasca et al. *1994*). **d** The follicles form a blunt elevation into the gut lumen, covered by a layer of epithelial cells, the follicular associated epithelium (FAE). ×100. Goblet cells are scant in the FAE

Lymphoreticular cell populations within the LFs

Immunohistochemistry was employed to identify and map the distribution of several classes of lymphocytes and antigen-presenting cells classically associated with sites of antigen sampling and presentation. Representative results are presented in Fig. 2. CD4+ T-lymphocytes were distributed predominantly in the parafollicular zones, although they were also observed within follicles (Fig. 2a). CD8+ lymphocytes were scantily scattered throughout follicles (Fig. 2b). A few WC1+ $\gamma\,\delta$ T-cells were observed infiltrating the lamina propria away from the follicle (Fig. 2c) but not in the follicle itself (not shown). CD2-positive cells ($\alpha\,\beta$ T-cells or natural killer cells) were common within the parafollicular region and a few were also present within follicles (Fig. 2d). Cells expressing CD21 (mature B-cells and follicular dendritic cells) were present in the light zone of the germinal centre of the LFs (Fig. 2e). CD40-expressing cells (B-cells or antigen-presenting cells) were present in the germinal centres of follicles (Fig. 2f). Some of these cells, presumably including antigen-presenting cells, were also observed in close proximity to the FAE. IgA- and IgM-expressing cells were present throughout the follicles and lamina propria indicating the presence of activated B-cells and plasma cells (not shown). MHC-class-II-expressing cells (not shown) were present on a large population of cells both within the follicles and in the parafollicular region. CD1b-dendritic cells were seen either scattered throughout the lamina propria or in small clusters in the proximity of LFs (not shown).

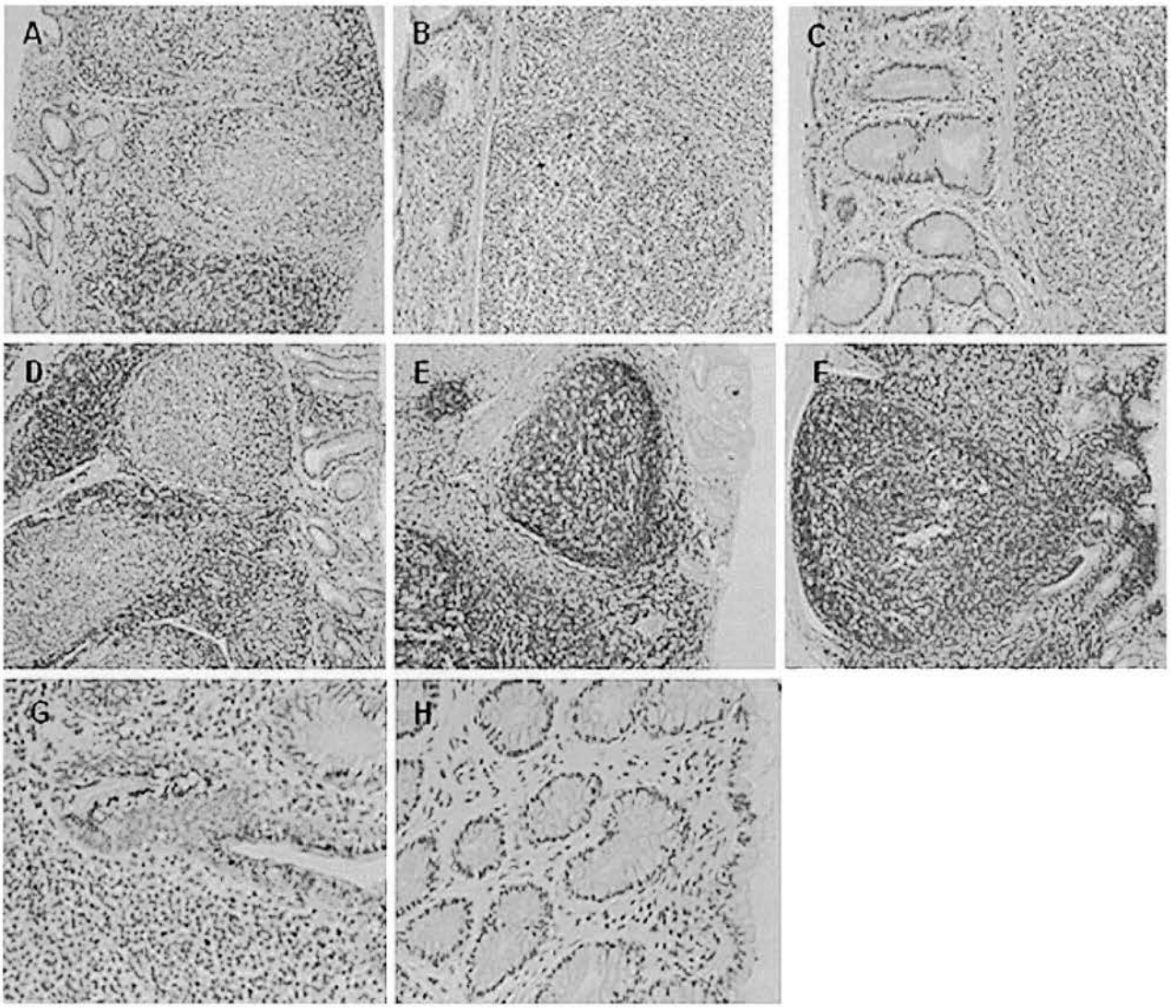

Fig. 2 Immunohistological analysis of cell surface antigens on lymphocytes associated with the LF of the bovine terminal rectal mucosa illustrating the distribution of (**a**) T-helper cells (CD4), (**b**) cytotoxic T-cells (CD8), (**c**) WC1+ $\gamma\delta$ T-cells, (**d**) $\alpha\beta$-T cells and natural killer cells (CD2), (**e**) mature B-cells and follicular dendritic cells (CD21), and (**f**) B-cells and antigen-presenting cells (CD40). B-lymphocytes were found mainly within the follicles and T-lymphocytes were distributed in the parafollicular region. ×250. The FAE and cells in the crypt in proximity to the follicle stained strongly for vimentin (**g**), whereas only a few cells in the crypts distant from the follicle (**h**) showed weak staining for vimentin. ×400

Characteristics of FAE

The FAE was characterised by immunohistochemical staining for vimentin, an intermediate filament of the cytoskeleton. The majority of cells within the FAE and follicle-associated crypts from the bovine terminal rectum stained strongly for vimentin (Fig. 2g); in these regions, typically >60% of epithelial cells were positive. Vimentin expression is a characteristic of M-cells, but not of other epithelial cell types, thus suggesting that a substantial proportion of FAE is composed of M-cells. Epithelium not associated with LFs (in this instance, rectal mucosa obtained from approximately 20 cm proximal to the RAJ) did not express vimentin, although occasional areas were weakly stained (Fig. 2h). In all

immunoreactive cells, vimentin was localised predominantly in the perinuclear cytoplasm of the cells. Many of the vimentin-positive cells appeared to contain basolateral indentation(s) enclosing lymphocytes (another characteristic of M-cells).

The follicle-rich area of the terminal rectum exhibited a heterogeneous surface morphology with a mixed cell population, as seen by SEM and TEM (Fig. 3). In the region immediately overlying the LFs, the majority of FAE cells were characterised by either short sparse irregular microvilli indicative of M-cells or fused/flattened microfolds and prominent invaginations of the basolateral membrane encircling leukocytes (Fig. 3a–d). Many cells in this region were distinct from the surrounding epithelium and displayed characteristics typical of bovine M-cells (Torres-Medina *1981*; Liebler et al. *1988*). The epithelium adjacent to FAE also showed heterogeneity, was interspersed with M-cells and predominated by an array of polygonal epithelial cells with regular long uniform densely-packed microvilli typical of enterocytes. The epithelium not associated with follicles consisted of a homogeneous enterocyte population with long uniform microvilli.

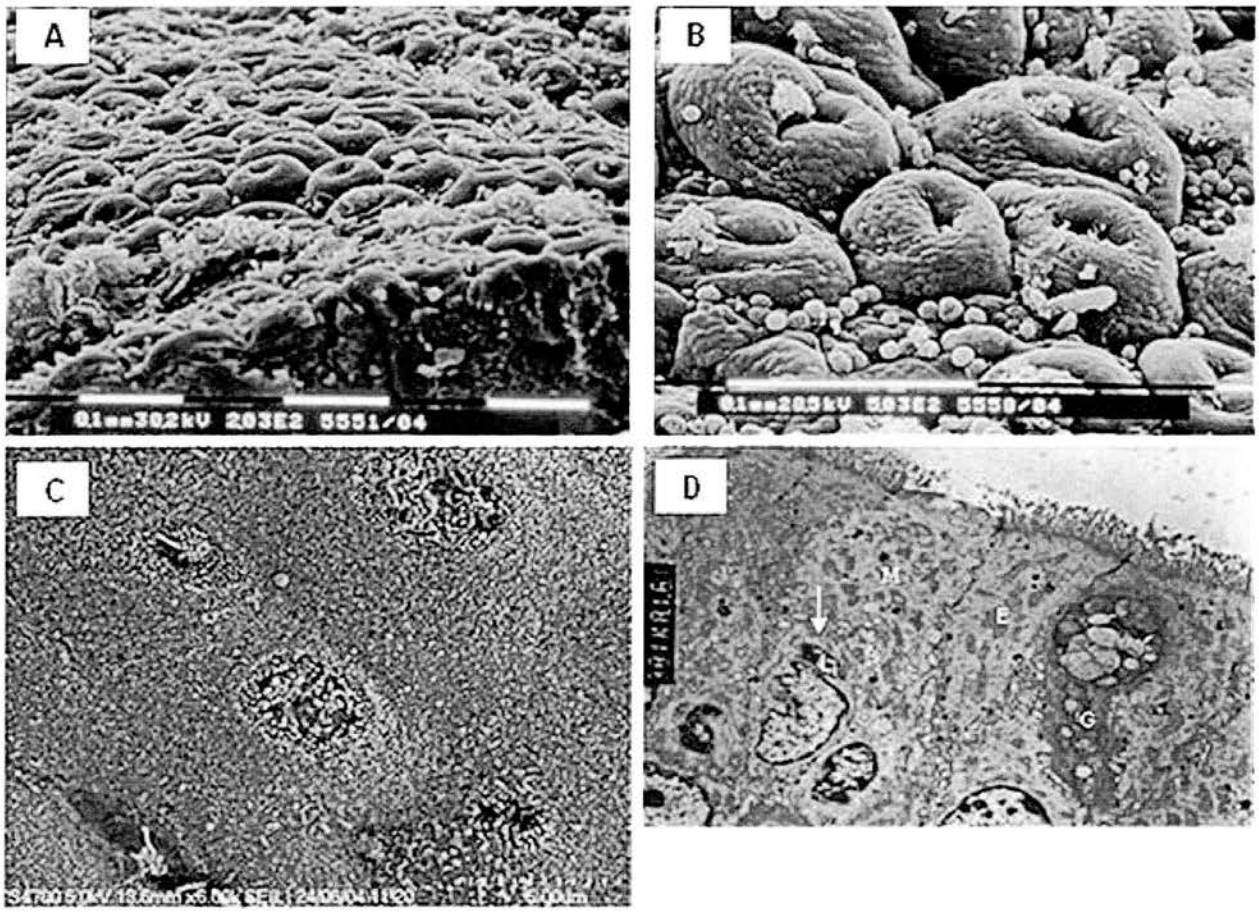

Fig. 3 Ultrastructure of FAE in the bovine terminal rectal mucosa. **a** Low magnification SEM image showing the lumenal surface of follicle-associated crypts. ×250. **b**, **c** Higher magnification SEM images of crypts showing heterogeneous cell populations, predominantly expressing short microvilli-M-like cells. ×600, ×6,000, respectively. **d** TEM showing M-cell (*M*) with stunted or fused microvilli with a leucocyte (*L*) in an intra-epithelial pocket on the basolateral surface. Note adjacent enterocyte (*E*) and goblet (*G*) cells. ×2,950

Lectin binding by FAE

Although various lectins have allowed the FAE to be distinguished in other animals (Table 2), none of the lectins used in this study specifically labelled the FAE of the bovine terminal rectum. Each lectin was tested at a range of dilutions on tissue fixed by three different methods (paraformaldehyde, ZSF or cryo-fixation). Depending upon concentration, the lectins either did not label epithelium at all or labelled all epithelial cell types with a similar intensity. This staining pattern was consistent with that from on-going investigations of sheep intestinal tissue (J. Huntley, unpublished) examined with a panel of 30 lectins (including UEA, consistently specific for mouse and rabbit M-cells). Hence, this result is not unique to cattle but reflects the absence of a consistent glycoconjugate to differentiate M-cells and highlights the crucial need for such M-cell markers. Lectin staining of murine Peyer 's patch tissue demonstrated UEA binding to the surface of cells of the dome epithelium (data not shown; A.C. Stanley, personal communication), thus validating our protocols and confirming that the absence of lectin staining was characteristics of bovine (and ovine) FAE.

Microparticle uptake by FAE

To characterise the cells in this region further, an in vitro and in vivo functional assay specific for the transcytosis activity of M-cells was performed. Tissue explants comprising FAE from the regions 0–3 cm or 18–20 cm cranial to the RAJ were cultured as described in the Materials and methods. Fluorescent latex beads were incubated with explants for 45 min as, during this period, tissue explants retained an intact functional mucosal epithelium as observed here and in previous investigations (Jackson et al. *2004*). Fluorescent confocal microscopy revealed microparticles present on the apical surface of FAE (Fig. 4a), whereas microparticles could not be detected in association with the absorptive epithelium, either adjacent to FAE or from mucosa 20 cm proximal to the RAJ. This finding was consistent with the localisation of M-cells to FAE as shown by immunohistochemical staining for vimentin described above. Uptake of microparticles by some cells within the FAE (Fig. 4b) was also demonstrated by confocal microscopy; the optical sectioning of cells showed fluorescent particles subjacent to the apical actin cytoskeleton (stained with phalloidin). There was no apparent difference in the cell association and uptake of particles of different sizes (0.2 and 0.5 μm). In vivo, particle uptake from the rectal lumen was evident from

45 min post-challenge and was restricted to the dome epithelium (Fig. 4c) of the rectum. At the later time point (60 min), beads were observed within the submucosal region (Fig. 4d), indicative of microparticle transcytosis.

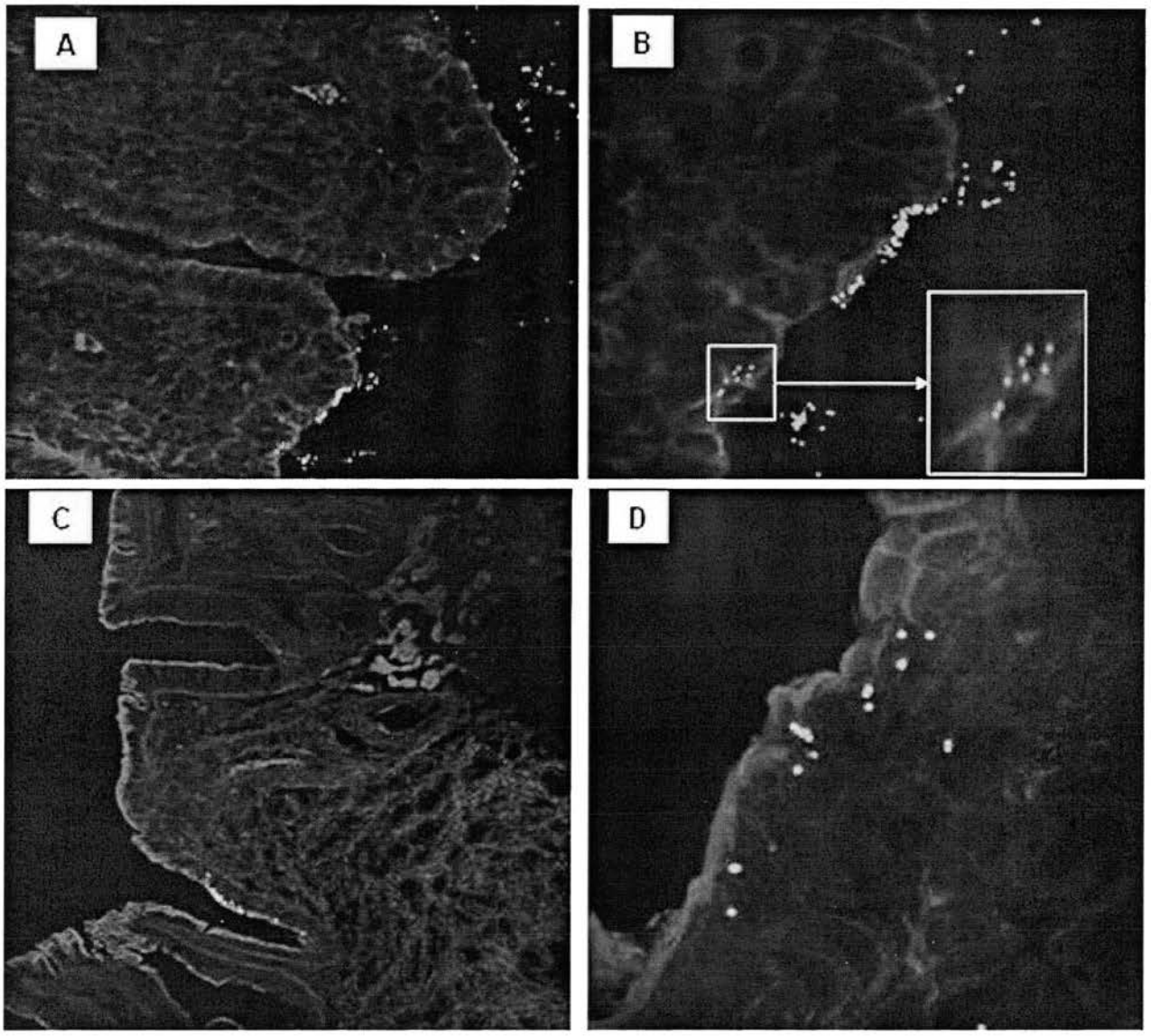

Fig. 4 Microparticle uptake by rectal tissue explants (**a**, **b**) or rectal FAE in situ in calves (**c**, **d**). Cryosections demonstrate adsorption and transport of 0.5-μm fluorescent latex microspheres (*green*). Tissues were counter-stained with phalloidin-TRITC (*red*) to visualise the cellular cytoskeleton. Tissue samples from approximately 45 min post-challenge (representative of results at 45–60 min post-challenge). Microspheres adhered to and were taken up by a subset cells in the FAE, a characteristic of M-cells. **a**, **c** Acquired with ×20 lens.s **b**, **d** Acquired with ×40 and ×63 lens, respectively. *Inset* in **b**: Higher magnification image showing particle internalisation. ×63 (zoom factor 2). For this, a series of optical sections were acquired on the Leica TCS NT confocal system and visualised in the Surpass module of the Imaris software suite (Bitplane, Zurich).

Discussion

LFs and the overlying FAE form discernable structures in the intestinal mucosa and have important functions in generating immune responses to mucosal pathogens. In the current investigation, LFs have been found to form compact clusters at high density in the region 0–

3 cm proximal to the RAJ of cattle, whereas solitary discrete follicles are present in more proximal sites in the rectum; no follicles have been observed at more proximal sites. Similar to our findings, patches of lymphoid tissue have been shown to be distributed around the circumference of the anal canal in other mammals including humans (Gebbers et al. *1992*; Langman and Rowland *1992*) and sheep (Sedgmen et al. *2002*) with the LFs more frequently being present in the submucosa than in the lamina propria as observed here. Therefore, rectal lymphoid tissues are similar in several animal species and, hence, cattle can be used as a representative mammal in which to characterise the cell populations within this region in order to assess the potential of this tissue as an immune-inductive site.

The immune-inductive role is dependent upon the presence and function of cell populations necessary for antigen sampling and processing and the induction of specific immune responses, including those involving M-cells, antigen-processing cells (APCs) and B- and T-lymphocytes. The immunophenotyping of LFs underlying rectal FAE has revealed distinct T-lymphocyte (CD4+, CD8+, $\gamma\delta$TCR+), B-lymphocyte (CD40, CD21, CD25), dendritic cell (CD1b) and IgA/IgM staining patterns within and around follicles. Thus, LFs in the rectum resemble those from other sites (Nagi and Babiuk *1988*; Sedgmen et al. *2002*; Gutierrez et al. *1999*), with the APC and lymphocyte populations necessary for the development of adaptive immune responses. Specialised epithelial cells involved in antigen sampling normally overlie LFs and these cells differ from enterocytes in morphology, function and other characteristics. Ultrastructural examination of the dome epithelium overlying follicles has revealed that FAE consists of more than one cell type. Numerous cells with short and sparse microvilli (indicative of M-cells) lie adjacent to cells showing uniform long microvilli (indicative of enteroabsorptive cells). The FAE possesses no, or only occasional, goblet cells, although this cell type becomes evident in the transitional region from the FAE to mucosal villous epithelium. M-cells represent a specialised epithelial cell population that samples lumenal macromolecular and particulate antigens for delivery to underlying APCs in LFs (Owen *1999*).

Specific glycoconjugates have been widely used to mark the apical membrane of M-cells (Gebert and Hach *1993*; Giannasca et al. *1999*; Gebert and Posselt *1997*; Clark et al. *1993*). However, with the exceptions of rabbit, mouse and other selected examples (see Table 2), no lectin has been identified that can be used as a common marker for these cells from different intestinal regions or in different animal species. The present investigation supports this conclusion, since none of the lectins has exhibited specificity for either M-cells or the FAE in the terminal rectum of cattle, an observation consistent with the FAE of sheep (J. Huntley, unpublished) in which a panel of 30 lectins has also failed to identify FAE-/M-cell-specific glycoconjugates. A great diversity of expression of M-cell-specific glycoconjugates has been observed both between animals and between tissues, e.g. UEA-1 is a marker for M-cells in Peyer's patches but not in the colon (Giannasca et al. *1994*) of mice (confirmed in our own studies; data not shown; A.C. Stanley, personal communication). Glycoconjugates characteristic of, and suitable for distinguishing, cattle (or sheep) FAE or M-cells remain to be defined and the current study highlights the crucial need for consistent M-cell markers to aid in the further characterisation of M-cell biology.

M-cells also express characteristic intermediate filaments, which are absent in absorptive enterocytes. Vimentin and cytokeratins 8 and 18 are found in rabbit, rat and pig intestinal M-cells (Gebert et al. *1992*; Jepson et al. *1992*; Rautenberg et al. *1996*) but are absent in enterocytes. In this study, we have observed strong staining for vimentin in the majority of cells of the FAE in the rectal mucosa and the majority of epithelial cells in crypts in close proximity to LFs. This staining pattern in dome-associated crypts resembles that observed

previously in rabbits (Lelouard et al. *2001*). The stained cells may be undifferentiated or precursor M-cells in the crypts adjacent to FAE (Gebert et al. *1999*). Vimentin staining is absent in epithelium not associated with follicles, such as the mucosa 20 cm proximal to the RAJ.

Although the considerable variation in the histochemistry of M-cells makes it difficult to characterise these cells fully, they can be defined by their key function, i.e. particulate/macromolecular antigen uptake and transcytosis, a characteristic that has been defined both in vivo and in vitro by using microparticle-uptake assays (Porta et al. *1992*; Ermak et al. *1995*; Pappo and Ermak *1989*; Landsverik *1988*). In this study, a functional assessment of M-cells has been carried out in tissue explants from terminal rectal mucosa and by in vivo calf challenges. Both 0.2 and 0.5 μm microparticles adhere to, and are internalised by, cells in the FAE. Other epithelial cells, within both rectal FAE and mucosa 20 cm proximal to the RAJ, show no microparticle attachment or uptake activity. The localisation of this function to rectal FAE correlates with the immunohistochemical detection of vimentin-expressing cells, thus supporting the conclusion that these cells do indeed represent M-cells and are involved in antigen sampling and delivery to rectal LFs via which specific immune responses are induced.

The finding that not all cells of the FAE bind and internalise microparticles, despite the majority of these cells being vimentin-positive, is intriguing and of possible functional significance. The ontogeny of M-cells is still a matter of much debate. Whether the subsets of vimentin-expressing cells in the rectal epithelium represent different stages in M-cell development or even different lineages remains to be defined, as does their involvement in immune-response induction. The aim of this study has not been directly to address whether M-cells represent a distinct lineage or an additional epithelial cell phenotype. However, we conclude that the juxtaposition of epithelium and LFs is an important determinant in triggering M-cell phenotype in both FAE and adjacent follicle-associated crypts. Lymphotoxin has been suggested as one possible effector (Debard et al. *1999*), although further study is required to elucidate the signals responsible for regulating M-cell development.

In summary, this investigation has demonstrated that the FAE overlying LFs in terminal rectum is predominated by cells expressing vimentin, a feature of M-cells. A subset of these cells binds and internalises microparticles, a key characteristic of M-cells. Importantly, antigen-presenting cells and lymphocytes occur within LFs of this site. Both of these cell types are necessary for the development of specific immune responses. Although additional investigations are required to broaden the characterisation of (1) the FAE cell subsets, (2) the cellular determinants involved in FAE interactions with pathogens and (3) the role of this site in the development of immune responses, the current investigation has demonstrated that the terminal rectum possesses the cellular populations characteristic of mucosal immune-inductive sites and that it may thus play a role in the development of mucosal immune responses.

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